noveon. 8EHQ-0404-15550 MR# 274665

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Health, Safety & Environmental

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April 12, 2004

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Attention: TSCA Section 8(e) Coordinator
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
1200 Constitution Avenue, N.W.
Washington, DC 20460-0001

Re: TSCA 8(e) Submission of Benzyl Benzoate, Benzyl Salicylate, and Alpha-amylcinnamaldehyde Algal Inhibition Studies

Dear Sir or Madam:

Noveon, Inc. (Noveon) submits this letter pursuant to Section 8(e) of the Toxic Substance Act (TSCA) to inform EPA of the findings of algal inhibition studies on benzyl benzoate, benzyl salicylate, alpha-amylcinnamaldehyde, and nonanal. Noveon has not made a determination as to whether a significant risk of injury to health or the environment is actually presented by the findings.

Benzyl benzoate (CAS# 120-51-4), benzyl salicylate (CAS# 118-58-1), alpha-amylcinnamaldehyde (CAS# 122-40-7), and nonanal (CAS# 124-19-6) were tested by the Flavor and Fragrance High Production Volume Consortia's in accordance with its commitment to the EPA High Production Volume Chemical Program. The acute toxicity studies indicate that these chemicals are highly toxic to the freshwater alga, *Selenastrum capricornutum*. In view of these findings, Noveon has elected to inform the EPA.

The final reports are enclosed.

None of the information in this submission is claimed as confidential business information.

If you have any questions, please contact Dr. Robert K. Hinderer at 216-447-5181 or robert.hinderer@noveoninc.com.

Sincerely,

Kenneth J. Willings
Vice President HS&E

cc: Tim Adams, Ph.D. (FFHPVC)
Robert K. Hinderer, Ph.D.

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Study Title

Growth and Reproduction Toxicity Test with Benzyl Benzoate and the Freshwater Alga, *Selenastrum capricornutum*

Guideline Number

OECD 201

Sponsor

The Flavor and Fragrance High Production Volume Consortia
1620 I Street, N.W.
Suite 925
Washington, DC 20006

Authors

Timothy J. Ward
Derek C. Wyskiel
Robert L. Boeri

Study Initiated

February 20, 2003

Study Completed

November 21, 2003

Testing Facility

T.R. Wilbury Laboratories, Inc.
40 Doaks Lane
Marblehead, Massachusetts 01945

I. GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was performed in compliance with OECD (1997) Good Laboratory Practice Standards. Characterization of the test substance was not conducted in compliance with GLP rules.

11-21-03

Derek C. Wyskiel — Date
Study Director

11/21/03

Test Facility Management — Date

II. QUALITY ASSURANCE STATEMENT

Submitted by: T.R. Wilbury Laboratories, Inc.
40 Doaks Lane
Marblehead, Massachusetts 01945

T.R. Wilbury Laboratories, Inc., study number 2459-FF, "Growth and Reproduction Limit Toxicity Test with Benzyl Benzoate and the Freshwater Alga, *Selenastrum capricornutum*," was audited by the T.R. Wilbury Quality Assurance Unit for compliance with the protocol, standard operating procedures, and applicable Good Laboratory Practices (OECD, 1997). Quality assurance audits were performed and the findings reported to the T.R. Wilbury Laboratories management and the study director on the following dates:

	Audit Date	Reported to Study Director	Reported to Management
Protocol:	29MAY02	29MAY02	29MAY02
In-life:	11APR03	14APR03	14APR03
	28APR03	30APR03	30APR03
Raw data/Draft:	18APR03	18APR03	18APR03
	08MAY03	08MAY03	08MAY03
Final report:	21NOV03	21NOV03	21NOV03

21-Nov-03

Arthur P. Paradice, RQAP-GLP
Quality Assurance Officer

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V. SUMMARY

The acute toxicity of benzyl benzoate to the freshwater alga, *Selenastrum capricornutum*, is described in this report. The test was performed for 72 hours from April 28 to May 1, 2003, at T.R. Wilbury Laboratories, Inc., in Marblehead, Massachusetts. It was conducted for The Flavor and Fragrance High Production Volume Consortia according to the protocol developed for T.R. Wilbury Study Number 2459-FF. Benzyl benzoate was supplied by International Flavors and Fragrances, Hazlet, New Jersey.

The test was performed under static conditions in sealed containers from which all air space had been removed to minimize the potential loss of test substance from test solutions to the atmosphere. The test was run with six concentrations of test substance at 24 ± 2°C. Nominal concentrations of benzyl benzoate were 0 mg/L (control), 0.033, 0.065, 0.13, 0.25, 0.50, and 1.0 mg/L. Initial measured concentrations of benzyl benzoate were: ND (none detected at or above the limit of quantitation; control), 0.0332, 0.0647, 0.127, 0.247, 0.472, and 0.972 mg/L. These measured concentrations, which ranged from 94 to 101% of the nominal concentrations, were used to calculate median effective concentrations (EC50s). Final measured concentrations ranged from <1 to 58% of nominal concentrations. Insoluble material was not observed at any tested concentration during the test.

The dilution water was sterile freshwater AAP medium adjusted to a pH of 7.5 ± 0.1. The test was performed in clear glass 40 mL vials that were filled to capacity to eliminate any head space and sealed with Teflon-lined caps. Algae used in the test were from a culture originally obtained from the Culture Collection of Algae of the University of Texas at Austin and acclimated to test conditions for more than 14 days at T.R. Wilbury Laboratories. Algae were distributed among twenty replicates of each control and 11 replicates of each treatment at the rate of approximately 10,000 cells/mL.

Exposure of algae to benzyl benzoate for 72 hours resulted in a median effective concentration (EC50) of 0.475 mg/L when calculated using the average specific growth rate, 0.363 mg/L when calculated using the number of cells/mL, and 0.311 mg/L when calculated using the area under the growth curve. The 72 hour no observed effect concentration (NOEC) is 0.247 mg/L benzyl benzoate when determined using the number of cells/mL or the average specific growth rate, and 0.0647 mg/L when determined using the area under the growth curve.

At the conclusion of the definitive toxicity test, a 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (the 0.972 mg/L benzyl benzoate concentration) was combined with fresh media. This culture was incubated under test conditions for 96 hours. During this period the number of algal cells increased from an initial calculated concentration of approximately 260 cells/mL to 820,000 cells/mL, indicating that the toxic effects were algistatic rather than algicidal.

VI. GENERAL INFORMATION

Material Tested:	Benzyl Benzoate
CAS Number:	120-51-4
Lot Number:	297835
Percent Active Ingredient:	99-100%
Study Initiation Date:	February 20, 2003 (protocol signed)
Experimental Start Date:	February 24, 2003
Experimental Termination Date:	May 5, 2003
Study Completion Date:	November 21, 2003
Archive Location -- biological raw data and a copy of the final report:	T.R. Wilbury Laboratories, Marblehead, MA
original final report	Transferred to Sponsor

VII. OBJECTIVE

The purpose of this study was to determine the 24, 48, and 72 hour median effective concentration (EC50) values and the 72 hour no observed effect concentration (NOEC) of the test substance to algae exposed under static conditions.

VIII. METHODS AND MATERIALS

A. TEST SUBSTANCE

The sample of benzyl benzoate used during the study (T.R. Wilbury sample number 1755) was delivered on February 11, 2003. It was contained in a 500 mL amber glass bottle that was labeled with the following information: "021200, Benz Benzoate, Lot 297835, X5861, 247, 12/19/02, 500 gr."

Benzyl benzoate (a clear liquid) was shipped from International Flavors and Fragrances, 600 Highway 36, Hazlet, New Jersey, 07730, at ambient temperature. The purity of the test substance was reported to be 99.0 to 100.0% active ingredient and the test substance was used as-received. Prior to use the test substance was stored at room temperature in the dark. All unused test substance is returned to the sponsor. The stability of the test substance under test conditions (aqueous solutions in sealed containers with no head space) was determined by the analysis of samples during the definitive toxicity test.

B. DILUTION WATER

Water used for acclimation of test organisms and for all toxicity testing was sterile freshwater AAP medium (U.S. EPA, 1978; T.R. Wilbury Standard Operating Procedure number 6) at a pH of 7.5 ± 0.1. Characterization of a representative sample of deionized water used to formulate media is presented in Table 1 and a description of the media is presented in Table 2.

C. TEST ORGANISM

Algae used for the test (*Selenastrum capricornutum*, UTEX 1648) were from a culture originally procured from the Culture Collection of Algae at the University of Texas at Austin and delivered to T.R. Wilbury Laboratories on July 17, 2001. The culture was transferred to sterile enriched media identical to media used for this test and maintained at test conditions for at least 14 days before the definitive test.

During the acclimation period, the culture was actively growing in at least 2 subcultures prior to the start of the toxicity test. The subsample of algae used to inoculate media at the start of the definitive test came from a seven day old culture. Identification of the culture .organisms, which are also referred to as *Raphidocelis subcapitata* and *Pseudokirchneriella subcapitata*, was verified using an appropriate taxonomic key.

Table 1. Chemical characterization of a representative sample of deionized water used to formulate dilution water for the toxicity test with benzyl benzoate and the freshwater alga, *Selenastrum capricornutum*.

Parameter[1]	Unit of Measurement	Detection Limit	Measured Value
Metals			
Aluminum	mg/L	0.01	0.02
Arsenic	mg/L	0.0004	ND[2]
Boron	mg/L	0.002	ND
Cadmium	mg/L	0.0001	ND
Calcium	mg/L	0.5	ND
Chromium	mg/L	0.0004	ND
Cobalt	mg/L	0.0001	0.0034
Copper	mg/L	0.0006	ND
Iron	mg/L	0.005	ND
Lead	mg/L	0.0001	0.001
Magnesium	mg/L	0.01	ND
Mercury	mg/L	0.0001	ND
Nickel	mg/L	0.0001	ND
Potassium	mg/L	0.1	ND
Silver	mg/L	0.0002	ND
Sodium	mg/L	0.5	ND
Zinc	mg/L	0.002	0.010
Chloride	mg/L	0.1	ND
Fluoride	mg/L	0.05	ND
Total Phosphorus	mg/L	0.02	ND
Total Organic Carbon	mg/L	0.5	ND
Organochlorine Pesticides	µg/L	4.0	ND
Toxaphene	µg/L	10.0	ND
Methoxychlor	µg/L	10.0	ND
Mirex	µg/L	10.0	ND
Organophosphorus Pesticides	µg/L	0.33	ND
PCBs	µg/L	0.05	ND

[1] Parameters were measured in deionized water (used to formulated dilution water) that was collected on December 24, 2002 (chloride and fluoride samples were collected on December 23, 2002) and analyzed by Enviro-Test Laboratories as part of routine water quality testing conducted twice per year.

[2] ND = not detected at or above the method detection limit.

Table 2. Description of the freshwater AAP medium used for the toxicity test with benzyl benzoate and the freshwater alga, *Selenastrum capricornutum*.

	Nutrient[1]	Final Concentration in Algal Medium (mg/L)
Macronutrients	$NaNO_3$	25.5
	$MgCl_2 \cdot 6H_2O$	12.2
	$CaCl_2 \cdot 2H_2O$	4.4
	$MgSO_4 \cdot 7H_2O$	14.7
	K_2HPO_4	1.044
	$NaHCO_3$	15.0
Micronutrients	H_3BO_3	0.1855
	$CoCl_2 \cdot 6H_2O$	0.0014
	$MnCl_2 \cdot 4H_2O$	0.4154
	$Na_2MoO_4 \cdot 2H_2O$	0.0073
	$ZnCl_2$	0.0033
	$Na_2EDTA \cdot 2H_2O$	0.30
	$FeCl_2 \cdot 6H_2O$	0.1598
	$CuCl_2 \cdot 2H_2O$	0.000012

[1] Each of the nutrients was prepared as a sterile stock solution in deionized water at 1,000 times the concentrations in the table. Algal medium was prepared by the addition of 1 mL of each stock solution to 1,000 mL of sterile deionized water.

D. TOXICITY TESTING

The study was performed according to T.R. Wilbury Study Protocol 2459-FF (Growth and Reproduction Limit Toxicity Test with Benzyl Benzoate and the Freshwater Alga, *Selenastrum capricornutum*), which was based on procedures of the OECD (1984). It was conducted for The Flavor and Fragrance High Production Volume Consortia and the Sponsor's Representative was Dr. Timothy B. Adams.

A range-finding test was conducted from February 24 to 27, 2003 with a control and two concentrations of benzyl benzoate. At the conclusion of the test, the number of cells/mL in the 10 mg/L test vessel was 11% of the number of cells/mL in the control flask and the number of cells/mL in the 100 mg/L test vessel was less than 1% of the number of cells/mL in the control flask. A second range-finding test was conducted from February 28 to March 3, 2003 with a control and three concentrations of benzyl benzoate. At the conclusion of the test, the number of cells/mL in the 0.10 and 1.0 mg/L test vessels was at least 60% of the number of cells/mL in the control flask, and the number of cells/mL in the 10 mg/L test vessel was less than 1% of the number of cells/mL in the control flask.

A third range-finding test was conducted from March 3 to 6, 2003 with a control and three concentrations of benzyl benzoate. At the conclusion of the test, the number of cells/mL in the 0.01, 0.10, and 1.0 mg/L test vessels was at least 62% of the number of cells/mL in the control flask. A fourth range-finding test was conducted from March 11 to 14, 2003 with a control and three concentrations of benzyl benzoate. At the conclusion of the test, the number of cells/mL in the 0.10 test vessel was 84% of the number of cells/mL in the control flask, and the number of cells/mL in the 0.50, 1.0, 5.0, and 10 mg/L test vessels was 14% or less of the number of cells/mL in the control flask. A definitive test was conducted from April 8 to 11, 2003 with a control and five concentrations of benzyl benzoate. At the conclusion of the test, the number of cells/mL in the 0.0330, 0.0758, 0.126, 0.242, and 0.482 mg/L (initial measured concentrations) test vessels averaged 89, 107, 77, 45, and 30% of the number of cells/mL in the control flask, respectively. The test was determined to be unacceptable because it was not conducted at a high enough concentration to allow the calculation of a 72-hour EC50 value.

The final definitive toxicity test was conducted from April 28 to May 1, 2003. It was performed at 24 ± 2°C with six concentrations of test substance and a control. A 10 mg/L stock solution was prepared on April 27, 2003 by weighing 0.0100 g of test substance into a 1,000 mL Class A glass volumetric flask and adjusting the volume of dilution water to the line. This stock solution was mixed overnight on a magnetic stirrer at room temperature. A series of solutions was prepared on April 28, 2003, by bringing 3.3, 6.5, 13, 25, 50, and 100 mL of the 10 mg/L stock solution to 1,000 mL with dilution water. Nominal concentrations of benzyl benzoate were 0 mg/L (control), 0.033, 0.065, 0.13, 0.25, 0.50, and 1.0 mg/L. A portion of each solution was transferred into a 1.0 L glass beaker and a 1.0 L portion of dilution water was also transferred to a glass beaker to serve as a control. The pH of the solutions was adjusted to 7.5 ± 0.1 with 0.1 N hydrochloric acid

(VWR Lot # 1190), if required. Water quality measurements were made and each solution was inoculated with approximately 10,000 algal cells/mL.

Solutions were subdivided into 11 clear glass 40 mL vials for each treatment (the control was subdivided into 20 replicates) and the vials, which were filled to capacity to eliminate any head space, were sealed with Teflon-lined caps. Test vessels were randomly arranged on a rotary shaker adjusted to approximately 100 rpm in an incubator during the test (a random numbers table was used to select the location for each vessel). A 24 hour light and 0 hour dark photoperiod was automatically maintained with cool-white fluorescent lights that provided a light intensity of approximately 400 to 410 footcandles (approximately 54 to 55 $\mu Ein/m^2sec$).

The number of algal cells/mL in each test vessel and the occurrence of relative size differences, unusual cell shapes, colors, flocculations, adherence of cells to test containers, or aggregation of cells was determined visually by means of direct microscopic examination with a hemocytometer. At 24, 48, and 72 hours, three treatment vessels and six control vessels were randomly selected and sacrificed (opened to the atmosphere) to allow daily determination of the number of algal cells/mL. The remaining two vessels at each concentration were used for the determination of benzyl benzoate concentration at the end of the test.

Temperature of the incubator was measured and recorded daily (thermometer number 2968) and the temperature in a representative vessel of water incubated with the test vessels was continuously recorded. The pH of test solutions was measured and recorded in the single solution of each concentration prior to its distribution to test vessels at the beginning of the test, and in all test vessels used for the determination of the number of algal cells/mL at the end of the test.

A 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (0.972 mg/L initial measured benzyl benzoate concentration) was combined in a 250-mL flask with 100 mL of fresh media to determine whether toxic effects were algicidal or algistatic. This culture was incubated under test conditions for 96 hours.

ANALYTICAL METHODS:

Analytical samples (20 mL) were collected into 40 mL glass VOA vials containing 1 drop of concentrated H_3PO_4. Samples collected at the start of the toxicity test were collected from test solutions just prior to the addition of algae and distribution of the test solutions to sealed test vessels. Samples collected at the end of the toxicity test were centrifuged to remove algal cells prior to acidification from pooled replicate test vessels. Samples outside the calibration range were diluted into the range with 0.1% H_3PO_4 in dilution water. An aliquot of sample was transferred into an autosampler vial and analyzed by HPLC/UV (Hewlett Packard 1100 Series HPLC). Typical analytical conditions were:

Column:	Zorbax 300SB-C3, 50 x 2.1 mm, 5 μm
Column Temperature:	40°C
Column Flow Rate:	1.5 mL/minute
Run Time:	10 minutes
UV Detector Wavelength:	220 nm
Injection Volume:	100 μL
Retention Time:	4.9 ± 0.5 minutes
Mobile Phase:	0.1% H_3PO_4 in 25/75 acetonitrile//HPLC water
Run:	Isocratic

HPLC chromatographic quantitation was achieved using a standard curve obtained from peak heights of injections of seven linearity standards: 0.0200, 0.0280, 0.0500, 0.100, 0.250, 0.500, and 0.800 mg/L. Standards were prepared by serial dilution of a 1,000 mg/L standard prepared in acetonitrile. The measured concentrations of benzyl benzoate in test samples were determined using the following equation:

$$\text{Concentration of Analyte} = \frac{\text{Sample Response - Curve Intercept}}{\text{Slope}} \times \text{Dilution Factor}$$

The analytical method was validated in triplicate at 0.020, 1.0, and 20 mg/L in a representative dilution water. Measured concentrations for samples with a nominal concentration of 0.020 mg/L averaged 0.0214 ± 0.001 mg/L before centrifugation to remove any undissolved test substance and 0.0209 ± 0.002 mg/L after centrifugation. Measured concentrations for samples with a nominal concentration of 1.0 mg/L averaged 1.00 ± 0.02 mg/L before centrifugation and 0.945 ± 0.017 mg/L after centrifugation. Measured concentrations for samples with a nominal concentration of 20 mg/L averaged 18.5 ± 1.0 mg/L before centrifugation and 17.2 ± 0.6 mg/L after centrifugation.

The limit of quantitation (LOQ) was calculated as ten times the mean sample height converted to concentration using the amount/height of the lowest concentration calibration standard, also incorporating the concentration factor for the control samples. The LOQ during the definitive test was 0.00165 mg/L. The limit of detection (LOD) is calculated as three times the mean sample height converted to concentration using the amount/height of the lowest concentration calibration standard. The LOD during the definitive test was 0.000496 mg/L.

E. STATISTICAL METHODS

The average specific growth rate was calculated as the natural log of the number of cells/mL at time t_1 minus the natural log of the number of cells/mL at time t_0 divided by the exposure period. The area under the growth curve was calculated using the following formula:

$$\text{Area} = \frac{N_1-N_0}{2} \times t_1 + \frac{N_1+N_2-2N_0}{2} \times (t_2-t_1) + \frac{N_{n-1}+N_n-2N_0}{2} \times (t_n-t_{n-1})$$

where:

N_0 = nominal number of cells/mL at time t_0

N_1 = measured number of cells/mL at time t_1

N_n = measured number of cells/mL at time t_n

t_1 = time of first measurement after the beginning of the test

t_n = time of n^{th} measurement after the beginning of the test

The 24, 48, and 72 hour EC50 values were calculated using the weighted least squares non-linear regression estimation procedure (Bruce and Versteeg, 1992). The slope of the concentration-response curve could not be calculated by this method. The no observed effect concentration (NOEC) was determined using a one-way analysis of variance (ANOVA) and Bonferroni's's test (TOXSTAT 3.3; Gulley, et al., 1990). The effective concentrations and NOECs were determined using the initial measured concentration of benzyl benzoate and the number of cells/mL, average specific growth rate, and area under the growth curve.

IX. RESULTS

Insoluble material was not observed during the test. Nominal concentrations of benzyl benzoate were: 0 mg/L (control), 0.033, 0.065, 0.13, 0.25, 0.50, and 1.0 mg/L. Initial measured concentrations of benzyl benzoate were: ND (none detected at or above the limit of quantitation; control), 0.0332, 0.0647, 0.127, 0.247, 0.472, and 0.972 mg/L. These initial measured concentrations ranged from 94 to 101% of nominal concentrations. Final measured concentrations were <1 to 58% of the nominal concentrations, indicating that once the aqueous solutions of benzyl benzoate were sealed into the test vessels with the algae, concentrations decreased during the 72-hour exposure period.

The algal population grew at an acceptable rate in the sealed vessels with no head space, resulting in an average of 245,000 cells/mL in the control after 72 hours (Table 4). No effects (relative size differences, unusual cell shapes, colors, flocculations, adherence of cells to test containers, or aggregation of cells) were noted during the test. Water quality throughout the test was within acceptable limits. The incubator temperature ranged from 24.0 to 24.3°C (Table A.1). The pH was not affected by the test substance (Table A.2).

Results of the exposure of algae to benzyl benzoate for 72 hours are presented in Tables 4, 5, and 6, and cell growth during the test is illustrated in Figure 1. The 24, 48, and 72 hour EC50 values are presented in Table 7. Exposure of *Selenastrum capricornutum* to benzyl benzoate for 72 hours resulted in an EC50 of 0.475 mg/L when calculated using the average specific growth rate, 0.363 mg/L when calculated using the number of cells/mL, and 0.311 mg/L when calculated using the area under the growth curve. The 72 hour NOEC is 0.247 mg/L benzyl benzoate when determined using the number of cells/mL or the average specific growth rate, and 0.0647 mg/L when determined using the area under the growth curve.

At the conclusion of the definitive toxicity test, a 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (the vessels with an initial measured concentration of 0.972 mg/L benzyl benzoate) was combined with 100 mL of fresh media in a 250-mL flask. This culture was incubated under test conditions for 96 hours. During this period the number of algal cells increased from an initial calculated concentration of 260 cells/mL to 820,000 cells/mL, indicating that the toxic effects were algistatic rather than algicidal.

Table 3. Measured concentrations of benzyl benzoate in test media during the toxicity test with the freshwater alga, *Selenastrum capricornutum*.

Nominal Concentration of Benzyl Benzoate (mg/L)	Measured Concentration of Benzyl Benzoate (mg/L)			
	0 hour	Percent of Nominal	72 hour	Percent of Nominal
0 (control)	ND[1]	--	ND	
0.033	0.0332	101	0.00692[4]	21
0.065	0.0647	100	0.0146	22
0.13	0.127	98	0.0447	34
0.25	0.247	99	0.0872	35
0.50	0.472	94	0.291	58
1.0	0.972	97	<0.00845	--
Laboratory Control Samples[2]				
0.25	0.248	99	0.252	101
	0.258	103	0.244	98
Stability Blank[3]				
1.0	0.951	95	0.934	93
Blank				
0	ND	--	ND	--

[1] ND = none detected at or above the limit of quantitation.
[2] Standards prepared in dilution water.
[3] Standard prepared in dilution water without algae and incubated with the test vessels.
[4] Estimated value; below the limit of quantitation but above the limit of detection.

Table 4. Cell growth data from the toxicity test with benzyl benzoate and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Benzyl Benzoate (mg/L)	Replicate[2]	Number of Algal Cells/mL[1] Hour of Exposure 0	24	48	72
Control	1	10,000	60,000	174,000	224,000
	2	10,000	52,000	154,000	256,000
	3	10,000	76,000	140,000	264,000
	4	10,000	54,000	140,000	266,000
	5	10,000	51,000	146,000	222,000
	6	10,000	66,000	172,000	240,000
	mean	10,000	60,000	154,000	245,000
0.0332	1	10,000	58,000	184,000	404,000
	2	10,000	49,000	190,000	430,000
	3	10,000	60,000	174,000	396,000
	mean	10,000	56,000	183,000	410,000
	% control	100	93	119	167
0.0647	1	10,000	72,000	190,000	316,000
	2	10,000	56,000	166,000	288,000
	3	10,000	60,000	160,000	382,000
	mean	10,000	63,000	172,000	329,000
	% control	100	105	112	134
0.127	1	10,000	58,000	124,000	304,000
	2	10,000	60,000	118,000	196,000
	3	10,000	58,000	114,000	240,000
	mean	10,000	59,000	119,000	247,000
	% control	100	98	77	101

[1] Cell counts at 0 hour are calculated based on the cell density of the culture used to inoculate the test vessels. Cell counts from 24 through 72 hours were made using a hemocytometer.

[2] Replicates (20 controls and 11 per treatment) were changed at each interval.

Table 4. Cell growth data from the toxicity test with benzyl benzoate and the freshwater alga, *Selenastrum capricornutum* (continued).

Initial Measured Concentration of Benzyl Benzoate (mg/L)	Replicate[2]	Number of Algal Cells/mL[1] Hour of Exposure 0	24	48	72
0.247	1	10,000	42,000	80,000	246,000
	2	10,000	39,000	68,000	254,000
	3	10,000	44,000	80,000	206,000
	mean	10,000	42,000	76,000	235,000
	% control	100	70	49	96
0.472	1	10,000	33,000	68,000	144,000
	2	10,000	36,000	58,000	110,000
	3	10,000	32,000	64,000	98,000
	mean	10,000	34,000	63,000	117,000
	% control	100	57	41	48
0.972	1	10,000	31,000	42,000	22,000
	2	10,000	33,000	41,000	12,000
	3	10,000	38,000	29,000	18,000
	mean	10,000	34,000	37,000	17,000
	% control	100	57	24	7

[1] Cell counts at 0 hour are calculated based on the cell density of the culture used to inoculate the test vessels. Cell counts from 24 through 72 hours were made using a hemocytometer.

[2] Replicates (20 controls and 11 per treatment) were changed at each interval.

Table 5. Average specific growth rate and percent of control from the toxicity test with benzyl benzoate and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Benzyl Benzoate (mg/L)		Average Specific Growth Rate Hour of Exposure 24	48	72
Control	mean	0.075	0.057	0.044
0.0332	mean	0.072	0.061	0.052
	% control	96	107	118
0.0647	mean	0.077	0.059	0.049
	% control	103	104	111
0.127	mean	0.074	0.052	0.045
	% control	99	91	102
0.247	mean	0.060	0.042	0.044
	% control	80	74	100
0.472	mean	0.051	0.038	0.034
	% control	68	67	77
0.972	mean	0.051	0.027	0.007
	% control	68	47	16

Table 6. Area under the growth curve and percent of control from the toxicity test with benzyl benzoate and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Benzyl Benzoate (mg/L)		Area Under the Growth Curve Hour of Exposure 24	48	72
Control	mean	600,000	2,928,000	7,476,000
0.0332	mean	552,000	3,180,000	10,056,000
	% control	92	109	135
0.0647	mean	636,000	3,216,000	8,988,000
	% control	106	110	120
0.127	mean	588,000	2,484,000	6,636,000
	% control	98	85	89
0.247	mean	384,000	1,560,000	5,052,000
	% control	64	53	68
0.472	mean	288,000	1,212,000	3,132,000
	% control	48	41	42
0.972	mean	288,000	900,000	1,308,000
	% control	48	31	17

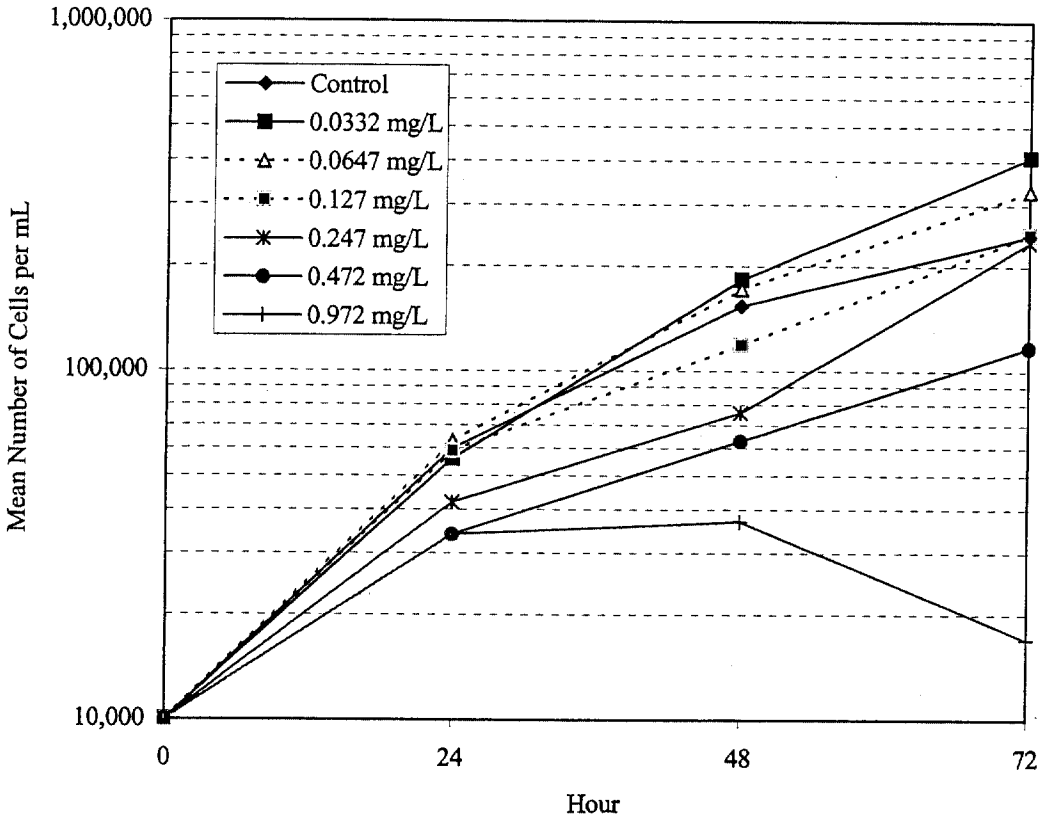


Figure 1. Growth of the freshwater alga, *Selenastrum capricornutum*, during the toxicity test with benzyl benzoate.

Table 7. Median effective concentrations (EC50s) and no observed effect concentrations (NOECs) from the toxicity test with benzyl benzoate and the freshwater alga, *Selenastrum capricornutum*.

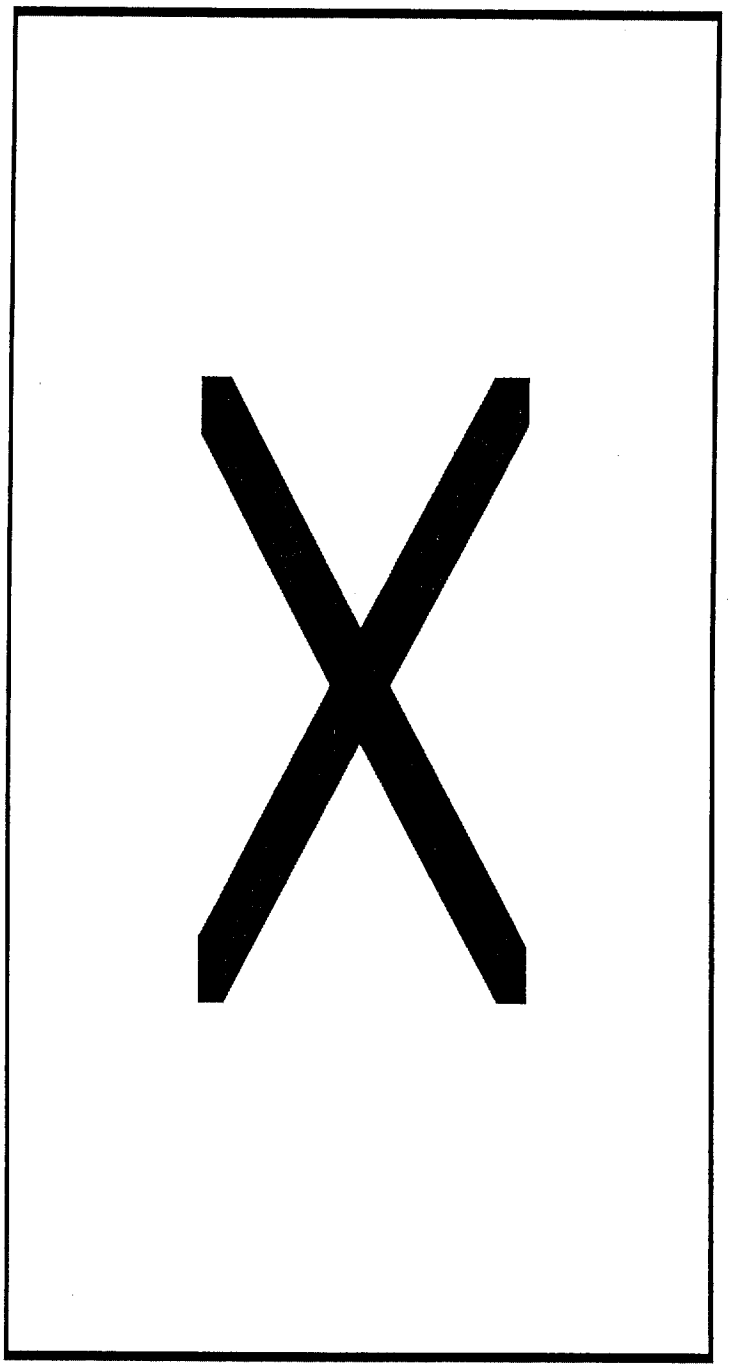
Time (hours)	Value (mg/L)[1]	95 Percent Confidence Limits (mg/L)[1]
Calculated Using the Number of Cells per Milliliter		
24 EC50	0.928	0.575 to >0.972
48 EC50	0.280	0.199 to 0.395
72 EC50	0.363	0.281 to 0.470
72 NOEC	0.247	
Calculated Using the Average Specific Growth Rate		
24 EC50	>0.972	--
48 EC50	0.787	0.648 to 0.955
72 EC50	0.475	0.474 to 0.477
72 NOEC	0.247	
Calculated Using the Area Under the Growth Curve		
24 EC50	0.617	0.383 to >0.972
48 EC50	0.337	0.244 to 0.465
72 EC50	0.311	0.246 to 0.392
72 NOEC	0.0647	

[1] Based on initial measured concentrations of benzyl benzoate.

X. PROTOCOL DEVIATIONS

No protocol deviations occurred.

XI. SIGNATURE PAGE



XII. REFERENCES

Bruce, R.D., and J.D. Versteeg. 1992. A Statistical Procedure for Modeling Continuous Toxicity Data. Environ. Toxicol. and Chem. Vol. 11. No. 10, pp. 1485-1494.

OECD. 1997. OECD Principles of Good Laboratory Practice. [C(97) 186 / Final].

OECD. 1984. OECD Guidelines for Testing of Chemicals. Section 2: Effects on Biotic Systems. Method 201, Alga Growth Inhibition Test. Adopted 7 June 1984.

U.S. EPA. 1978. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test. EPA-600/9-78-018. Environmental Research Laboratory, Corvallis, Oregon.

Appendix A. WATER QUALITY DATA FROM THE TOXICITY TEST

Table A.1. Temperatures measured during the toxicity test with benzyl benzoate and the freshwater alga, *Selenastrum capricornutum*.

Hour of Exposure	Temperature of Incubator (°C)
0	24.0
24	24.3
48	24.3
72	24.3

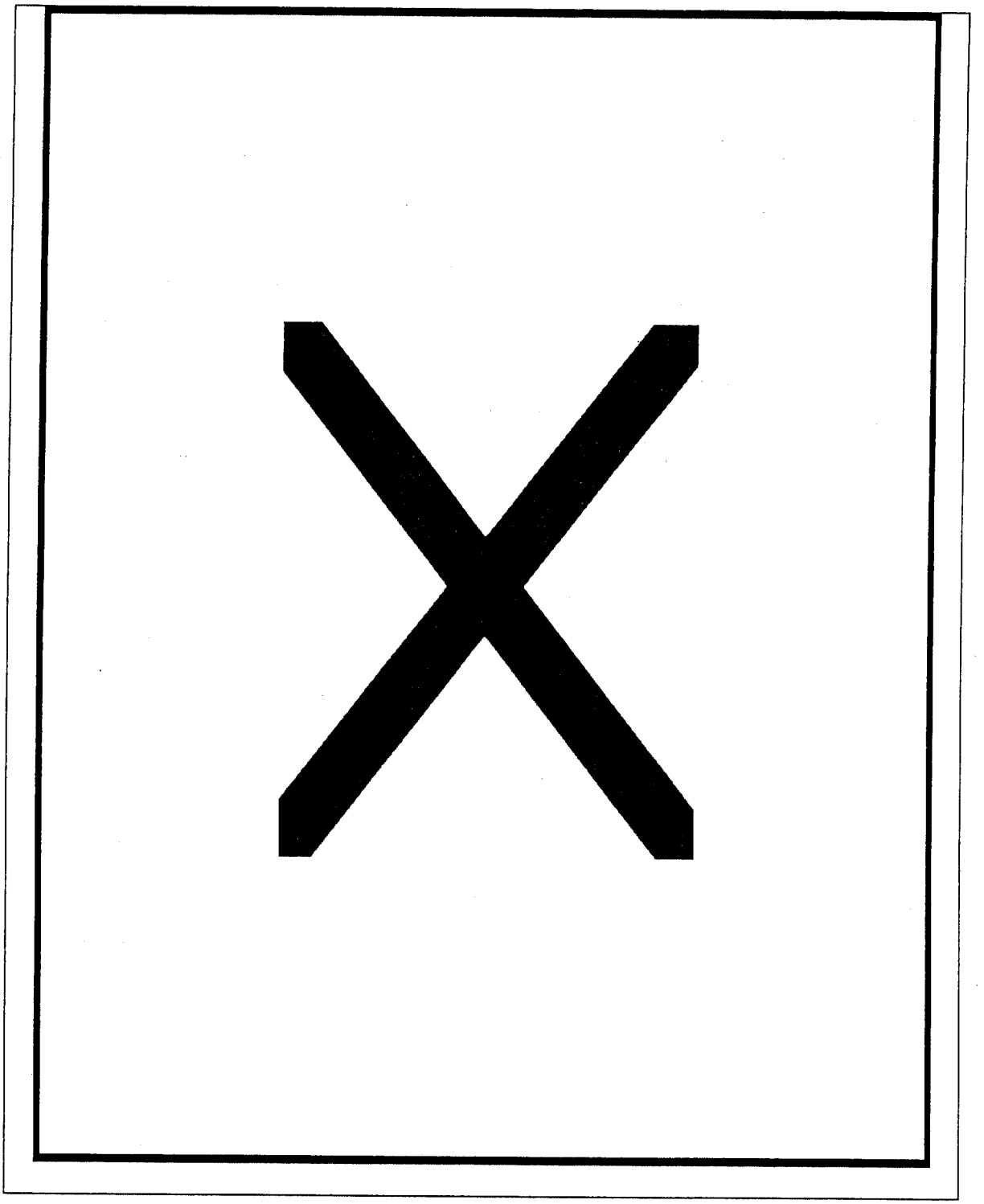
Table A.2. pH values measured during the toxicity test with benzyl benzoate and the freshwater alga, *Selenastrum capricornutum*.

Mean Measured Concentration of Benzyl Benzoate (mg/L)	Replicate[2]	pH	
		Initial	Final
Control	1	7.5	9.6
	2		9.6
	3		9.6
	4		9.7
	5		9.6
	6		9.7
0.0332	1	7.4	9.8
	2		9.8
	3		9.8
0.0647	1	7.5	9.9
	2		9.8
	3		9.9
0.127	1	7.5	9.8
	2		9.8
	3		9.9
0.247	1	7.4	9.9
	2		9.9
	3		9.9
0.472	1	7.5	9.0
	2		9.0
	3		9.0
0.972	1	7.5	8.7
	2		8.6
	3		8.6

[1] Initial pH measurements were made in stock solutions prior to their distribution into replicate test vessels. Final pH measurements were made in test vessel replicates used for

algal cell counting at 72 hours (6 of the 20 control vessels and three of the 11 vessels of each treatment).

Appendix B. CERTIFICATE OF ANALYSIS



MR#174665

Study Title

Growth and Reproduction Toxicity Test with Benzyl Salicylate and the Freshwater Alga, *Selenastrum capricornutum*

Guideline Number

OECD 201

Sponsor

The Flavor and Fragrance High Production Volume Consortia
1620 I Street, N.W.
Suite 925
Washington, DC 20006

Authors

Timothy J. Ward
Robert L. Boeri

Study Initiated

February 14, 2003

Study Completed

June 6, 2003

Testing Facility

T.R. Wilbury Laboratories, Inc.
40 Doaks Lane
Marblehead, Massachusetts 01945

I. GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was performed in compliance with OECD (1997) Good Laboratory Practice Standards. Characterization of the test substance was not conducted in compliance with GLP rules.

Robert L. Boeri — 06-06-05
Study Director — Date

Test Facility Management — Date

II. QUALITY ASSURANCE STATEMENT

Submitted by: T.R. Wilbury Laboratories, Inc.
40 Doaks Lane
Marblehead, Massachusetts 01945

T.R. Wilbury Laboratories, Inc., study number 2461-FF, "Growth and Reproduction Limit Toxicity Test with Benzyl Salicylate and the Freshwater Alga, *Selenastrum capricornutum*," was audited by the T.R. Wilbury Quality Assurance Unit for compliance with the protocol, standard operating procedures, and applicable Good Laboratory Practices (OECD, 1997). Quality assurance audits were performed and the findings reported to the T.R. Wilbury Laboratories management and the study director on the following dates:

	Audit Date	Reported to Study Director	Reported to Management
Protocol:	29MAY02	29MAY02	29MAY02
In-life:	04APR03	04APR03	04APR03
Raw data/Draft:	15APR03	15APR03	15APR03
Final report:	06JUN03	06JUN03	06JUN03

Arthur P. Paradice 06-JUN-03

Arthur P. Paradice, RQAP-GLP
Quality Assurance Officer

III. TABLE OF CONTENTS

IV. INDEX OF TABLES AND FIGURES

V. SUMMARY

The acute toxicity of benzyl salicylate to the freshwater alga, *Selenastrum capricornutum*, is described in this report. The test was performed for 72 hours from April 4 to 7, 2003, at T.R. Wilbury Laboratories, Inc., in Marblehead, Massachusetts. It was conducted for The Flavor and Fragrance High Production Volume Consortia according to the protocol developed for T.R. Wilbury Study Number 2461-FF. Benzyl salicylate was supplied by International Flavors and Fragrances, Hazlet, New Jersey.

The test was performed under static conditions in sealed containers from which all air space had been removed to minimize the potential loss of test substance from test solutions to the atmosphere. The test was run with five concentrations of test substance at 24 ± 2°C. Nominal concentrations of benzyl salicylate were 0 mg/L (control), 0.50, 1.0, 2.0, 4.0, and 8.0 mg/L. Initial measured concentrations of benzyl salicylate were: ND (none detected at or above the limit of quantitation; control), 0.502, 1.01, 1.92, 3.85, and 7.39 mg/L. These measured concentrations, which ranged from 92 to 101% of the nominal concentrations, were used to calculate median effective concentrations (EC50s). Final measured concentrations ranged from 4 to 68% of nominal concentrations. Insoluble material was not observed at any tested concentration during the test.

The dilution water was sterile freshwater AAP medium adjusted to a pH of 7.5 ± 0.1. The test was performed in clear glass 40 mL vials that were filled to capacity to eliminate any head space and sealed with Teflon-lined caps. Algae used in the test were from a culture originally obtained from the Culture Collection of Algae of the University of Texas at Austin and acclimated to test conditions for more than 14 days at T.R. Wilbury Laboratories. Algae were distributed among twenty replicates of each control and 11 replicates of each treatment at the rate of approximately 10,000 cells/mL.

Exposure of algae to benzyl salicylate for 72 hours resulted in a median effective concentration (EC50) of 0.691 mg/L when calculated using the average specific growth rate, 0.735 mg/L when calculated using the number of cells/mL, and 1.29 mg/L when calculated using the area under the growth curve. The 72 hour no observed effect concentration (NOEC) is 0.502 mg/L benzyl salicylate when determined using the number of cells/mL or the average specific growth rate, and <0.502 mg/L when determined using the area under the growth curve.

At the conclusion of the definitive toxicity test, a 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (the 3.85 mg/L and 7.39 mg/L benzyl salicylate concentrations) was combined with fresh media. These cultures were incubated under test conditions for 168 hours. During this period the number of algal cells increased from an initial calculated concentration of <10,000 cells/mL to 828,000 cells/mL at 3.85 mg/L and 274,000 cells/mL at 7.39 mg/L, indicating that the toxic effects were algistatic rather than algicidal.

VI. GENERAL INFORMATION

Material Tested:	Benzyl Salicylate
CAS Number:	118-58-1
Lot Number:	299171
Percent Active Ingredient:	99-100%
Study Initiation Date:	February 14, 2003 (protocol signed)
Experimental Start Date:	February 24, 2003
Experimental Termination Date:	April 14, 2003
Study Completion Date:	June 6, 2003
Archive Location -- biological raw data and a copy of the final report:	T.R. Wilbury Laboratories, Marblehead, MA
original final report	Transferred to Sponsor

VII. OBJECTIVE

The purpose of this study was to determine the 24, 48, and 72 hour median effective concentration (EC50) values and the 72 hour no observed effect concentration (NOEC) of the test substance to algae exposed under static conditions.

VIII. METHODS AND MATERIALS

A. TEST SUBSTANCE

The sample of benzyl salicylate used during the study (T.R. Wilbury sample number 1754) was delivered on February 11, 2003. It was contained in a 500 mL amber glass bottle that was labeled with the following information: "021540, Benz Sal, Lot 299171, X58761, 251, 12/19/02, 500 g."

Benzyl salicylate (a clear liquid) was shipped from International Flavors and Fragrances, 600 Highway 36, Hazlet, New Jersey, 07730, at ambient temperature. The purity of the test substance was reported to be 99.0 to 100.0% active ingredient and the test substance was used as-received. Prior to use the test substance was stored at room temperature in the dark. All unused test substance is returned to the sponsor. The stability of the test substance under test conditions (aqueous solutions in sealed containers with no head space) was determined by the analysis of samples during the definitive toxicity test.

B. DILUTION WATER

Water used for acclimation of test organisms and for all toxicity testing was sterile freshwater AAP medium (U.S. EPA, 1978; T.R. Wilbury Standard Operating Procedure number 6) at a pH of 7.5 ± 0.1. Characterization of a representative sample of deionized water used to formulate media is presented in Table 1 and a description of the media is presented in Table 2.

C. TEST ORGANISM

Algae used for the test (*Selenastrum capricornutum*, UTEX 1648) were from a culture originally procured from the Culture Collection of Algae at the University of Texas at Austin and delivered to T.R. Wilbury Laboratories on July 17, 2001. The culture was transferred to sterile enriched media identical to media used for this test and maintained at test conditions for at least 14 days before the definitive test.

During the acclimation period, the culture was actively growing in at least 2 subcultures prior to the start of the toxicity test. The subsample of algae used to inoculate media at the start of the definitive test came from an eight day old culture. Identification of the culture organisms, which are also referred to as *Raphidocelis subcapitata* and *Pseudokirchneriella subcapitata*, was verified using an appropriate taxonomic key.

Table 1. Chemical characterization of a representative sample of deionized water used to formulate dilution water for the toxicity test with benzyl salicylate and the freshwater alga, *Selenastrum capricornutum*.

Parameter[1]	Unit of Measurement	Detection Limit	Measured Value
Metals			
Aluminum	mg/L	0.01	0.02
Arsenic	mg/L	0.0004	ND[2]
Boron	mg/L	0.002	ND
Cadmium	mg/L	0.0001	ND
Calcium	mg/L	0.5	ND
Chromium	mg/L	0.0004	ND
Cobalt	mg/L	0.0001	0.0034
Copper	mg/L	0.0006	ND
Iron	mg/L	0.005	ND
Lead	mg/L	0.0001	0.001
Magnesium	mg/L	0.01	ND
Mercury	mg/L	0.0001	ND
Nickel	mg/L	0.0001	ND
Potassium	mg/L	0.1	ND
Silver	mg/L	0.0002	ND
Sodium	mg/L	0.5	ND
Zinc	mg/L	0.002	0.010
Chloride	mg/L	0.1	ND
Fluoride	mg/L	0.05	ND
Total Phosphorus	mg/L	0.02	ND
Total Organic Carbon	mg/L	0.5	ND
Organochlorine Pesticides	µg/L	4.0	ND
Toxaphene	µg/L	10.0	ND
Methoxychlor	µg/L	10.0	ND
Mirex	µg/L	10.0	ND
Organophosphorus Pesticides	µg/L	0.33	ND
PCBs	µg/L	0.05	ND

[1] Parameters were measured in deionized water (used to formulated dilution water) that was collected on December 24, 2002 (chloride and fluoride samples were collected on December 23, 2002) and analyzed by Enviro-Test Laboratories as part of routine water quality testing conducted twice per year.

[2] ND = not detected at or above the method detection limit.

Table 2. Description of the freshwater AAP medium used for the toxicity test with benzyl salicylate and the freshwater alga, *Selenastrum capricornutum*.

	Nutrient[1]	Final Concentration in Algal Medium (mg/L)
Macronutrients	$NaNO_3$	25.5
	$MgCl_2 \cdot 6H_2O$	12.2
	$CaCl_2 \cdot 2H_2O$	4.4
	$MgSO_4 \cdot 7H_2O$	14.7
	K_2HPO_4	1.044
	$NaHCO_3$	15.0
Micronutrients	H_3BO_3	0.1855
	$CoCl_2 \cdot 6H_2O$	0.0014
	$MnCl_2 \cdot 4H_2O$	0.4154
	$Na_2MoO_4 \cdot 2H_2O$	0.0073
	$ZnCl_2$	0.0033
	$Na_2EDTA \cdot 2H_2O$	0.30
	$FeCl_2 \cdot 6H_2O$	0.1598
	$CuCl_2 \cdot 2H_2O$	0.000012

[1] Each of the nutrients was prepared as a sterile stock solution in deionized water at 1,000 times the concentrations in the table. Algal medium was prepared by the addition of 1 mL of each stock solution to 1,000 mL of sterile deionized water.

D. TOXICITY TESTING

The study was performed according to T.R. Wilbury Study Protocol 2461-FF (Growth and Reproduction Limit Toxicity Test with Benzyl Salicylate and the Freshwater Alga, *Selenastrum capricornutum*), which was based on procedures of the OECD (1984). It was conducted for The Flavor and Fragrance High Production Volume Consortia and the Sponsor's Representative was Dr. Timothy B. Adams.

A range-finding test was conducted from February 24 to 27, 2003 with a control and two concentrations of benzyl salicylate. At the conclusion of the test, the number of cells/mL in the 10 and 100 mg/L test vessels was less than 1% of the number of cells/mL in the control flask. A second range-finding test was conducted from February 28 to March 3, 2003 with a control and three concentrations of benzyl salicylate. At the conclusion of the test, the number of cells/mL in the 0.10 and 1.0 mg/L test vessels was at least 82% of the number of cells/mL in the control flask, and the number of cells/mL in the 10 mg/L test vessel was less than 1% of the number of cells/mL in the control flask.

The definitive toxicity test was conducted from April 4 to 7, 2003. It was performed at 24 ± 2°C with five concentrations of test substance and a control. An 8.0 mg/L stock solution was prepared on April 3, 2003 by weighing 0.0160 g of test substance into a 2,000 mL Class A glass volumetric flask and adjusting the volume of dilution water to the line. This stock solution was mixed overnight on a magnetic stirrer at room temperature. A series of solutions was prepared on April 4, 2003, by bringing 63, 130, 250, 500, and 1,000 mL of the 8.0 mg/L stock solution to 1,000 mL with dilution water. Nominal concentrations of benzyl salicylate were 0 mg/L (control), 0.50, 1.0, 2.0, 4.0, and 8.0 mg/L. A portion of each solution was transferred into a 1.0 L glass beaker and a 1.0 L portion of dilution water was also transferred to a glass beaker to serve as a control. The pH of the solutions was adjusted to 7.5 ± 0.1 with 0.1 N hydrochloric acid (VWR Lot # 1190), if required. Water quality measurements were made and each solution was inoculated with approximately 10,000 algal cells/mL.

Solutions were subdivided into 11 clear glass 40 mL vials for each treatment (the control was subdivided into 20 replicates) and the vials, which were filled to capacity to eliminate any head space, were sealed with Teflon-lined caps. Test vessels were randomly arranged on a rotary shaker adjusted to approximately 100 rpm in an incubator during the test (a random numbers table was used to select the location for each vessel). A 24 hour light and 0 hour dark photoperiod was automatically maintained with cool-white fluorescent lights that provided a light intensity of approximately 390 footcandles (approximately 55 to 56 $\mu Ein/m^2sec$).

The number of algal cells/mL in each test vessel and the occurrence of relative size differences, unusual cell shapes, colors, flocculations, adherence of cells to test containers, or aggregation of cells was determined visually by means of direct microscopic examination with a hemocytometer. At 24, 48, and 72 hours, three treatment vessels and six control

vessels were randomly selected and sacrificed (opened to the atmosphere) to allow daily determination of the number of algal cells/mL. The remaining two vessels at each concentration were used for the determination of benzyl salicylate concentration at the end of the test.

Temperature of the incubator was measured and recorded daily (thermometer number 2968) and the temperature in a representative vessel of water incubated with the test vessels was continuously recorded. The pH of test solutions was measured and recorded in the single solution of each concentration prior to its distribution to test vessels at the beginning of the test, and in all test vessels used for the determination of the number of algal cells/mL at the end of the test.

A 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (3.85 and 7.39 mg/L benzyl salicylate concentrations) was combined in a 250-mL flask with 100 mL of fresh media to determine whether toxic effects were algicidal or algistatic. This culture was incubated under test conditions for 168 hours.

ANALYTICAL METHODS:

Analytical samples (20 mL) were collected into 40 mL glass VOA vials containing 1 drop of concentrated H_3PO_4. Samples collected at the start of the toxicity test were collected from test solutions just prior to the addition of algae and distribution of the test solutions to sealed test vessels. Samples collected at the end of the toxicity test were centrifuged to remove algal cells prior to acidification from pooled replicate test vessels. Samples outside the calibration range were diluted into the range with 0.1% H_3PO_4 in dilution water. An aliquot of sample was transferred into an autosampler vial and analyzed by HPLC/UV (Hewlett Packard 1100 Series HPLC). Typical analytical conditions were:

Column:	Zorbax 300SB-C3, 50 x 2.1 mm, 5 μm
Column Temperature:	40°C
Column Flow Rate:	1.5 mL/minute
Run Time:	10 minutes
UV Detector Wavelength:	220 nm
Injection Volume:	100 μL
Retention Time:	5.9 ± 0.5 minutes
Mobile Phase:	0.1% H_3PO_4 in 25/75 acetonitrile//HPLC water
Run:	Isocratic

HPLC chromatographic quantitation was achieved using a standard curve obtained from peak areas of injections of seven linearity standards: 0.250, 0.300, 0.500, 0.800, 1.00, 1.50, and 2.00, mg/L. Standards were prepared by serial dilution of a 1,000 mg/L standard prepared in acetonitrile. The measured concentrations of benzyl salicylate in test samples were determined using the following equation:

$$\text{Concentration of Analyte} = \frac{\text{Sample Response - Curve Intercept}}{\text{Slope}} \times \text{Dilution Factor}$$

The analytical method was validated in triplicate at 0.50 and 10 mg/L in a representative dilution water. Measured concentrations for samples with a nominal concentration of 0.50 mg/L averaged 0.494 ± 0.007 mg/L before centrifugation to remove any undissolved test substance and 0.485 ± 0.018 mg/L after centrifugation. Measured concentrations for samples with a nominal concentration of 10 mg/L averaged 9.84 ± 1.04 mg/L before centrifugation and 8.75 ± 0.173 mg/L after centrifugation.

The limit of quantitation (LOQ) was calculated as ten times the mean sample height converted to concentration using the amount/height of the lowest concentration calibration standard, also incorporating the concentration factor for the control samples. The LOQ during the definitive test was 0.00197 mg/L. The limit of detection (LOD) is calculated as three times the mean sample height converted to concentration using the amount/height of the lowest concentration calibration standard. The LOD during the definitive test was 0.000592 mg/L.

E. STATISTICAL METHODS

The average specific growth rate was calculated as the natural log of the number of cells/mL at time t_1 minus the natural log of the number of cells/mL at time t_0 divided by the exposure period. The area under the growth curve was calculated using the following formula:

$$\text{Area} = \frac{N_1-N_0}{2} \times t_1 + \frac{N_1+N_2-2N_0}{2} \times (t_2-t_1) + \frac{N_{n-1}+N_n-2N_0}{2} \times (t_n-t_{n-1})$$

where:

N_0 = nominal number of cells/mL at time t_0
N_1 = measured number of cells/mL at time t_1
N_n = measured number of cells/mL at time t_n
t_1 = time of first measurement after the beginning of the test
t_n = time of n^{th} measurement after the beginning of the test

The 24 hour EC50 values could not be calculated because growth was insufficient at all concentrations. The 48 and 72 hour EC50 values were calculated using the weighted least squares non-linear regression estimation procedure (Bruce and Versteeg, 1992). The slope of the concentration-response curve could not be calculated by this method.

The no observed effect concentration (NOEC) was determined using a one-way analysis of variance (ANOVA) and Bonferroni's's test (TOXSTAT 3.3; Gulley, et al., 1990). The effective concentrations and NOECs were determined using the initial measured concentration of benzyl salicylate and the number of cells/mL, average specific growth rate, and area under the growth curve. Concentrations that did not allow any cell growth (i.e. <10,000 cells/mL) were assumed to be different from the control and excluded from the calculation of the NOEC values.

IX. RESULTS

Insoluble material was not observed during the test. Nominal concentrations of benzyl salicylate were: 0 mg/L (control), 0.50, 1.0, 2.0, 4.0, and 8.0 mg/L. Initial measured concentrations of benzyl salicylate were: ND (none detected at or above the limit of quantitation; control), 0.502, 1.01, 1.92, 3.85, and 7.39 mg/L. These initial measured concentrations ranged from 92 to 101% of nominal concentrations. Final measured concentrations were 4 to 68% of the nominal measured concentrations, indicating that once the aqueous solutions of benzyl salicylate were sealed into the test vessels with the algae, concentrations decreased during the 72-hour exposure period.

The algal population grew at an acceptable rate in the sealed vessels with no head space, resulting in an average of 266,000 cells/mL in the control after 72 hours (Table 4). No effects (relative size differences, unusual cell shapes, colors, flocculations, adherence of cells to test containers, or aggregation of cells) were noted during the test. Water quality throughout the test was within acceptable limits. The incubator temperature ranged from 24.0 to 24.7°C (Table A.1). The pH was not affected by the test substance (Table A.2).

Results of the exposure of algae to benzyl salicylate for 72 hours are presented in Tables 4, 5, and 6, and cell growth during the test is illustrated in Figure 1. The 24, 48, and 72 hour EC50 values are presented in Table 7. Exposure of *Selenastrum capricornutum* to benzyl salicylate for 72 hours resulted in an EC50 of 0.691 mg/L when calculated using the average specific growth rate, 0.735 mg/L when calculated using the number of cells/mL, and 1.29 mg/L when calculated using the area under the growth curve. The 72 hour NOEC is 0.502 mg/L benzyl salicylate when determined using the number of cells/mL or the average specific growth rate, at <0.502 mg/L when determined using the area under the growth curve.

At the conclusion of the definitive toxicity test, a 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (the 3.85 mg/L and 7.39 mg/L benzyl salicylate concentrations) was combined with 100 mL of fresh media in a 250-mL flask. These cultures were incubated under test conditions for 168 hours. During this period the number of algal cells increased from an initial calculated concentration of <10,000 cells/mL to 828,000 cells/mL at 3.85 mg/L and 274,000 cells/mL at 7.39 mg/L, indicating that the toxic effects were algistatic rather than algicidal.

Table 3. Measured concentrations of benzyl salicylate in test media during the toxicity test with the freshwater alga, *Selenastrum capricornutum*.

Nominal Concentration of Benzyl Salicylate (mg/L)	Measured Concentration of Benzyl Salicylate (mg/L)			
	0 hour	Percent of Nominal	72 hour	Percent of Nominal
0 (control)	ND[1]	--	ND	
0.50	0.502	100	0.0249[3]	4
1.0	1.01	101	0.340	34
2.0	1.92	96	1.18	59
4.0	3.85	96	2.71	68
8.0	7.39	92	5.19	65
Laboratory Control Samples[2]				
2.0	1.92 1.93	97	2.11 2.13	106
Blank				
0	ND	--	ND	--

[1] ND = none detected at or above the limit of quantitation.
[2] Standards prepared in dilution water.

Table 4. Cell growth data from the toxicity test with benzyl salicylate and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Benzyl Salicylate (mg/L)	Replicate[2]	Number of Cells per Milliliter[1] Hour of Exposure 0	24	48	72
Control	1	10,000	10,000	186,000	294,000
	2	10,000	12,000	174,000	246,000
	3	10,000	10,000	104,000	274,000
	4	10,000	11,000	122,000	282,000
	5	10,000	17,000	130,000	236,000
	6	10,000	10,000	146,000	266,000
	mean	10,000	12,000	144,000	266,000
0.502	1	10,000	<10,000	100,000	298,000
	2	10,000	10,000	64,000	240,000
	3	10,000	<10,000	112,000	274,000
	mean	10,000	<10,000	92,000	271,000
	% control	100	<83	64	102
1.01	1	10,000	<10,000	32,000	74,000
	2	10,000	<10,000	28,000	78,000
	3	10,000	<10,000	17,000	84,000
	mean	10,000	<10,000	26,000	79,000
	% control	100	<83	18	30
1.92	1	10,000	10,000	<10,000	23,000
	2	10,000	<10,000	<10,000	22,000
	3	10,000	<10,000	<10,000	21,000
	mean	10,000	<10,000	<10,000	22,000
	% control	100	<83	<7	8
3.85	1	10,000	<10,000	<10,000	<10,000
	2	10,000	<10,000	<10,000	<10,000
	3	10,000	<10,000	<10,000	<10,000
	mean	10,000	<10,000	<10,000	<10,000
	% control	100	<83	<7	<4
7.39	1	10,000	<10,000	<10,000	<10,000
	2	10,000	<10,000	<10,000	<10,000
	3	10,000	<10,000	<10,000	<10,000
	mean	10,000	<10,000	<10,000	<10,000
	% control	100	<83	<7	<4

[1] Cell counts at 0 hour are calculated based on the cell density of the culture used to inoculate the test vessels. Cell counts from 24 through 72 hours were made using a hemocytometer.

[2] Replicates (20 controls and 11 per treatment) were changed at each interval.

Table 5. Average specific growth rate and percent of control from the toxicity test with benzyl salicylate and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Benzyl Salicylate (mg/L)		Average Specific Growth Rate		
		Hour of Exposure		
		24	48	72
Control	mean	0.008	0.056	0.046
0.502	mean	0.000	0.046	0.046
	% control	0	82	100
1.01	mean	0.000	0.020	0.029
	% control	0	36	63
1.92	mean	0.000	0.000	0.011
	% control	0	0	24
3.85	mean	0.000	0.000	0.000
	% control	0	0	0
7.39	mean	0.000	0.000	0.000
	% control	0	0	0

Table 6. Area under the growth curve and percent of control from the toxicity test with benzyl salicylate and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Benzyl Salicylate (mg/L)		Area Under the Growth Curve		
		Hour of Exposure		
		24	48	72
Control	mean	24,000	1,656,000	6,336,000
0.502	mean	0	984,000	5,100,000
	% control	0	59	80
1.01	mean	0	192,000	1,212,000
	% control	0	12	19
1.92	mean	0	0	144,000
	% control	0	0	2
3.85	mean	0	0	0
	% control	0	0	0
7.39	mean	0	0	0
	% control	0	0	0

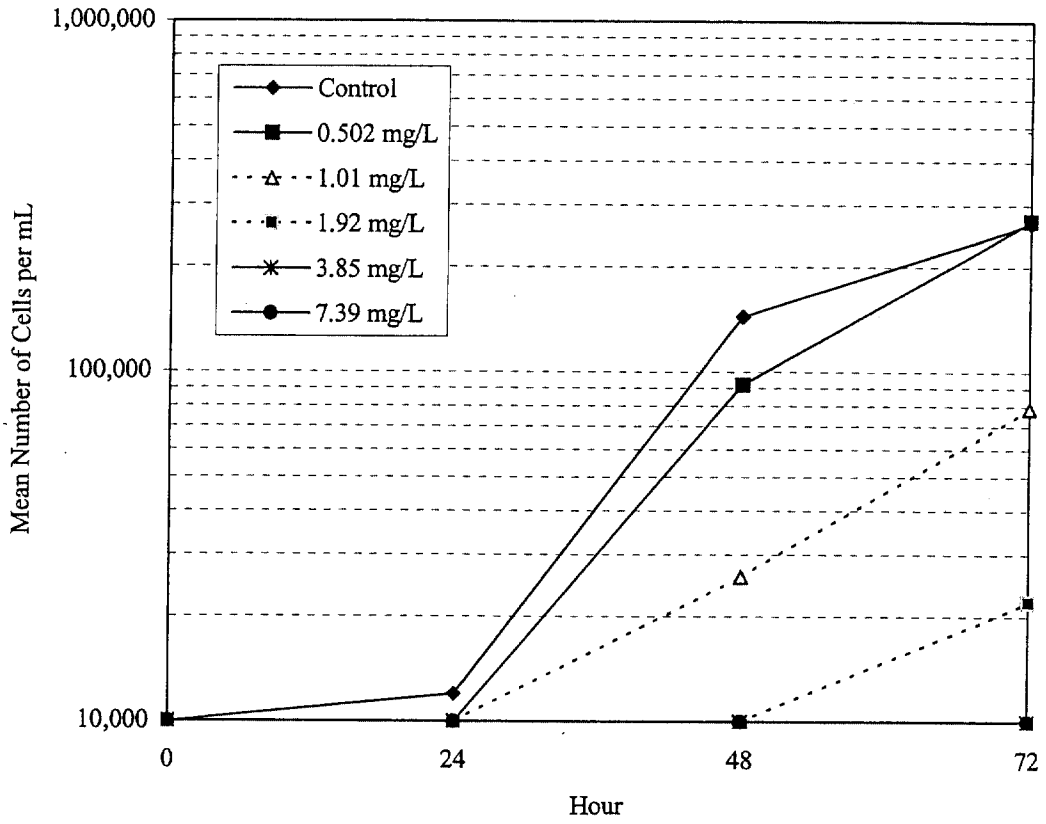


Figure 1. Growth of the freshwater alga, *Selenastrum capricornutum*, during the toxicity test with benzyl salicylate.

Table 7. Median effective concentrations (EC50s) and no observed effect concentrations (NOECs) from the toxicity test with benzyl salicylate and the freshwater alga, *Selenastrum capricornutum.*

Time (hours)	Value (mg/L)[1]	95 Percent Confidence Limits (mg/L)[1]
Calculated Using the Number of Cells per Milliliter		
48 EC50	--	--
72 EC50	0.735	--
72 NOEC	0.502	
Calculated Using the Average Specific Growth Rate		
48 EC50	0.978	--
72 EC50	1.29	1.18 to 1.41 mg/L
72 NOEC	0.502	
Calculated Using the Area Under the Growth Curve		
48 EC50	0.572	0.500 to 0.654
72 EC50	0.691	0.628 to 0.759
72 NOEC	<0.502	

[1] Based on initial measured concentrations of benzyl salicylate.

X. PROTOCOL DEVIATIONS

No protocol deviations occurred.

XI. SIGNATURE PAGE

06.06.03

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XII. REFERENCES

Bruce, R.D., and J.D. Versteeg. 1992. A Statistical Procedure for Modeling Continuous Toxicity Data. Environ. Toxicol. and Chem. Vol. 11. No. 10, pp. 1485-1494.

OECD. 1997. OECD Principles of Good Laboratory Practice. [C(97) 186 / Final].

OECD. 1984. OECD Guidelines for Testing of Chemicals. Section 2: Effects on Biotic Systems. Method 201, Alga Growth Inhibition Test. Adopted 7 June 1984.

U.S. EPA. 1978. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test. EPA-600/9-78-018. Environmental Research Laboratory, Corvallis, Oregon.

Appendix A. WATER QUALITY DATA FROM THE TOXICITY TEST

Table A.1. Temperatures measured during the toxicity test with benzyl salicylate and the freshwater alga, *Selenastrum capricornutum*.

Hour of Exposure	Temperature of Incubator (°C)
0	24.0
24	24.0
48	24.0
72	24.7

Table A.2. pH values measured during the toxicity test with benzyl salicylate and the freshwater alga, *Selenastrum capricornutum*.

Mean Measured Concentration of Benzyl Salicylate (mg/L)	Replicate[2]	pH	
		Initial	Final
Control	1	7.5	9.3
	2		9.5
	3		9.6
	4		9.7
	5		9.7
	6		9.6
0.502	1	7.5	9.8
	2		9.8
	3		9.7
1.01	1	7.5	8.5
	2		8.5
	3		8.5
1.92	1	7.4	7.5
	2		7.5
	3		7.5
3.85	1	7.4	7.4
	2		7.4
	3		7.4
7.39	1	7.4	7.4
	2		7.4
	3		7.4

[1] Initial pH measurements were made in stock solutions prior to their distribution into replicate test vessels. Final pH measurements were made in test vessel replicates used for algal cell counting at 72 hours (6 of the 20 control vessels and three of the 11 vessels of each treatment).

Appendix B. CERTIFICATE OF ANALYSIS

International Flavors & Fragrances Inc.

IFF

Certificate of Analysis

Ship To / Sold To:

Customer Order:
IFF Order / Item:
Delivery / Item:
Customer Number:

Info Text:

Material: **BENZYL SALICYLATE**
Flashpoint: **200° F**

IFF Batch: **299171**
Order Quantity: **1 OZ**

Date of Manufacture: **11/16/02**

Test	Test Data	Unit
Visual Color & Appearance	--- **VISCOLOR PASS**	
Primary Odor Evaluation	--- **ODOREVAL PASS**	
SPECIFIC GRAVITY @ D25/25	1.1800	gm/ml
SPECIFIC GRAVITY @ D20/20	1.1830	gm/ml
RELATIVE DENSITY @ D20/4	1.1809	#
REFRACTIVE INDEX @ nD25	1.5805	#
REFRACTIVE INDEX @ nD20	1.5825	#

John McGrogan
Quality Control Department

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MR# 274665

Study Title

Growth and Reproduction Toxicity Test with Alpha-Amylcinnamaldehyde and the Freshwater Alga, *Selenastrum capricornutum*

Guideline Number

OECD 201

Sponsor

The Flavor and Fragrance High Production Volume Consortia
1620 I Street, N.W.
Suite 925
Washington, DC 20006

Authors

Robert L. Boeri
Derek C. Wyskiel
Timothy J. Ward

Study Initiated

February 20, 2003

Study Completed

September 18, 2003

Testing Facility

T.R. Wilbury Laboratories, Inc.
40 Doaks Lane
Marblehead, Massachusetts 01945

I. GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was performed in compliance with OECD (1997) Good Laboratory Practice Standards. Characterization of the test substance was not conducted in compliance with GLP rules.

Derek C. Wyskiel
Study Director

9-18-03
Date

Test Facility Management

9/18/03
Date

II. QUALITY ASSURANCE STATEMENT

Submitted by: T.R. Wilbury Laboratories, Inc.
40 Doaks Lane
Marblehead, Massachusetts 01945

T.R. Wilbury Laboratories, Inc., study number 2462-FF, "Growth and Reproduction Toxicity Test with Alpha-Amylcinnamaldehyde and the Freshwater Alga, *Selenastrum capricornutum*," was audited by the T.R. Wilbury Quality Assurance Unit for compliance with the protocol, standard operating procedures, and applicable Good Laboratory Practices (OECD, 1997). Quality assurance audits were performed and the findings reported to the T.R. Wilbury Laboratories management and the study director on the following dates:

	Audit Date	Reported to Study Director	Reported to Management
Protocol:	29MAY02	29MAY02	29MAY02
In-life:	28MAY03	28MAY03	28MAY03
	02JUN03	03JUN03	03JUN03
Raw data/Draft:	12JUN03	12JUN03	12JUN03
Final report:	18SEP03	18SEP03	18SEP03

18-SEP-03
Arthur P. Paradice, RQAP-GLP
Quality Assurance Officer

III. TABLE OF CONTENTS

IV. INDEX OF TABLES AND FIGURES

V. SUMMARY

The acute toxicity of alpha-amylcinnamaldehyde to the freshwater alga, *Selenastrum capricornutum*, is described in this report. The test was performed for 72 hours from May 30 to June 2, 2003, at T.R. Wilbury Laboratories, Inc., in Marblehead, Massachusetts. It was conducted for The Flavor and Fragrance High Production Volume Consortia according to the protocol developed for T.R. Wilbury Study Number 2462-FF. Alpha-Amylcinnamaldehyde was supplied by International Flavors and Fragrances, Inc, 600 Highway 36, Hazlet, New Jersey.

The test was performed under static conditions in sealed containers from which all air space had been removed to minimize the potential loss of test substance from test solutions to the atmosphere. The test was run with six concentrations of test substance at 24 ± 2°C. Nominal concentrations of alpha-amylcinnamaldehyde were 0 mg/L (control), 0.095, 0.19, 0.38, 0.75, 1.5, and 3.0 mg/L. Initial measured concentrations of alpha-amylcinnamaldehyde were: ND (none detected at or above the limit of quantitation; control), 0.0934, 0.154, 0.363, 0.651, 1.39, and 2.75 mg/L. These initial measured concentrations, which ranged from 81 to 98% of the nominal concentrations, were used to calculate median effective concentrations (EC50s). Final measured concentrations ranged from less than 0.00259 mg/L to 0.0176 mg/L. Insoluble material was not observed at any tested concentration during the test.

The dilution water was sterile freshwater AAP medium adjusted to a pH of 7.5 ± 0.1. The test was performed in clear glass 40 mL vials that were filled to capacity to eliminate any head space and sealed with Teflon-lined caps. Algae used in the test were from a culture originally obtained from the Culture Collection of Algae of the University of Texas at Austin and acclimated to test conditions for more than 14 days at T.R. Wilbury Laboratories. Algae were distributed among twenty replicates of each control and 11 replicates of each treatment at the rate of approximately 10,000 cells/mL.

Exposure of algae to alpha-amylcinnamaldehyde for 72 hours resulted in a median effective concentration (EC50) of 1.88 mg/L when calculated using the average specific growth rate, 1.18 mg/L when calculated using the number of cells/mL, and 1.24 mg/L when calculated using the area under the growth curve. The 72 hour no observed effect concentration (NOEC) is 0.154 mg/L alpha-amylcinnamaldehyde when determined using the number of cells/mL, the average specific growth rate, or the area under the growth curve.

At the conclusion of the definitive toxicity test, a 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (the 2.75 mg/L alpha-amylcinnamaldehyde concentration) was combined with fresh media. This culture was incubated under test conditions for 120 hours. During this period the number of algal cells increased from an initial calculated concentration of approximately 300 cells/mL to approximately 140,000 cells/mL, indicating that the toxic effects were algistatic rather than algicidal.

VI. GENERAL INFORMATION

Material Tested:	Alpha-Amylcinnamaldehyde
Batch Number:	248084
Percent Active Ingredient:	100%
Study Initiation Date:	February 20, 2003 (protocol signed)
Experimental Start Date:	March 4, 2003
Experimental Termination Date:	June 7, 2003
Study Completion Date:	September 18, 2003
Archive Location -- biological raw data and a copy of the final report:	T.R. Wilbury Laboratories, Marblehead, MA
original final report	Transferred to Sponsor

VII. OBJECTIVE

The purpose of this study was to determine the 24, 48, and 72 hour median effective concentration (EC50) values and the 72 hour no observed effect concentration (NOEC) of the test substance to algae exposed under static conditions.

VIII. METHODS AND MATERIALS

TEST SUBSTANCE

The sample of alpha-amylcinnamaldehyde used during the study (T.R. Wilbury sample number 1753) was delivered on February 11, 2003. It was contained in a 500 mL amber glass bottle that was labeled with the following information: "014350 Amyl Cinn Ald, Coeur, Lot 248084, X58761, 248, 12/19/02, 500 gr."

Alpha-Amylcinnamaldehyde (a slightly yellow colored liquid) was shipped from International Flavors and Fragrances, Inc, 600 Highway 36, Hazlet, New Jersey, at ambient temperature. The purity of the test substance was reported to be 100% active ingredient and the test substance was used as-received. Prior to use the test substance was stored at room temperature in the dark. All unused test substance is returned to the sponsor. The stability of the test substance under test conditions (aqueous solutions in sealed containers with no head space) was determined by the analysis of samples during the definitive toxicity test.

DILUTION WATER

Water used for acclimation of test organisms and for all toxicity testing was sterile freshwater AAP medium (U.S. EPA, 1978; T.R. Wilbury Standard Operating Procedure number 6) at a pH of 7.5 ± 0.1. Characterization of a representative sample of deionized water used to formulate media is presented in Table 1 and a description of the media is presented in Table 2.

TEST ORGANISM

Algae used for the test (*Selenastrum capricornutum*, UTEX 1648) were from a culture originally procured from the Culture Collection of Algae at the University of Texas at Austin and delivered to T.R. Wilbury Laboratories on July 17, 2001. The culture was transferred to sterile enriched media identical to media used for this test and maintained at test conditions for at least 14 days before the definitive test.

During the acclimation period, the culture was actively growing in at least 2 subcultures prior to the start of the toxicity test. The subsample of algae used to inoculate media at the start of the definitive test came from an eight day old culture. Identification of the culture organisms, which are also referred to as *Raphidocelis subcapitata* and *Pseudokirchneriella subcapitata*, was verified using an appropriate taxonomic key.

Table 1. Chemical characterization of a representative sample of deionized water used to formulate dilution water for the toxicity test with alpha-amylcinnamaldehyde and the freshwater alga, *Selenastrum capricornutum*.

Parameter[1]	Unit of Measurement	Detection Limit	Measured Value
Metals			
Aluminum	mg/L	0.01	0.02
Arsenic	mg/L	0.0004	ND[2]
Boron	mg/L	0.002	ND
Cadmium	mg/L	0.0001	ND
Calcium	mg/L	0.5	ND
Chromium	mg/L	0.0004	ND
Cobalt	mg/L	0.0001	0.0034
Copper	mg/L	0.0006	ND
Iron	mg/L	0.005	ND
Lead	mg/L	0.0001	0.001
Magnesium	mg/L	0.01	ND
Mercury	mg/L	0.0001	ND
Nickel	mg/L	0.0001	ND
Potassium	mg/L	0.1	ND
Silver	mg/L	0.0002	ND
Sodium	mg/L	0.5	ND
Zinc	mg/L	0.002	0.010
Chloride	mg/L	0.1	ND
Fluoride	mg/L	0.05	ND
Total Phosphorus	mg/L	0.02	ND
Total Organic Carbon	mg/L	0.5	ND
Organochlorine Pesticides	µg/L	4.0	ND
Toxaphene	µg/L	10.0	ND
Methoxychlor	µg/L	10.0	ND
Mirex	µg/L	10.0	ND
Organophosphorus Pesticides	µg/L	0.33	ND
PCBs	µg/L	0.05	ND

[1] Parameters were measured in deionized water (used to formulated dilution water) that was collected on December 24, 2002 (chloride and fluoride samples were collected on December 23, 2002) and analyzed by Enviro-Test Laboratories as part of routine water quality testing conducted twice per year.

[2] ND = not detected at or above the method detection limit.

Table 2. Description of the freshwater AAP medium used for the toxicity test with alpha-amylcinnamaldehyde and the freshwater alga, *Selenastrum capricornutum*.

	Nutrient[1]	Final Concentration in Algal Medium (mg/L)
Macronutrients	$NaNO_3$	25.5
	$MgCl_2 \cdot 6H_2O$	12.2
	$CaCl_2 \cdot 2H_2O$	4.4
	$MgSO_4 \cdot 7H_2O$	14.7
	K_2HPO_4	1.044
	$NaHCO_3$	15.0
Micronutrients	H_3BO_3	0.1855
	$CoCl_2 \cdot 6H_2O$	0.0014
	$MnCl_2 \cdot 4H_2O$	0.4154
	$Na_2MoO_4 \cdot 2H_2O$	0.0073
	$ZnCl_2$	0.0033
	$Na_2EDTA \cdot 2H_2O$	0.30
	$FeCl_2 \cdot 6H_2O$	0.1598
	$CuCl_2 \cdot 2H_2O$	0.000012

[1] Each of the nutrients was prepared as a sterile stock solution in deionized water at 1,000 times the concentrations in the table. Algal medium was prepared by the addition of 1 mL of each stock solution to 1,000 mL of sterile deionized water.

TOXICITY TESTING

The study was performed according to T.R. Wilbury Study Protocol 2462-FF (Growth and Reproduction Toxicity Test with Alpha-Amylcinnamaldehyde and the Freshwater Alga, *Selenastrum capricornutum*), which was based on procedures of the OECD (1984). It was conducted for The Flavor and Fragrance High Production Volume Consortia and the Sponsor's Representative was Dr. Timothy B. Adams.

A range-finding test was conducted from March 4 to 7, 2003 with a control and four concentrations of alpha-amylcinnamaldehyde. At the conclusion of the test, the percent of control growth was as follows; 0.10 mg/L = 83%, 1.0 mg/L = 46%, 10 and 100 mg/L = <1%. A second range-finding test was conducted from March 11 to 14, 2003 with a control and five concentrations of alpha-amylcinnamaldehyde. At the conclusion of the test, the percent of control growth was as follows; 0.050 mg/L = 127%, 0.10 mg/L = 92%, 0.50 mg/L = 67%, 1.0 mg/L = 6%, and 5.0 mg/L = <1%.

A definitive toxicity test was attempted from May 27 to 29, 2003 with a control and five nominal concentrations of alpha-amylcinnamaldehyde (0.065, 0.13, 0.25, 0.50, and 1.0 mg/L). The test was terminated after 48 hours due to poor analytical recoveries in the sample collected at the start of the test.

The final definitive toxicity test was conducted from May 30 to June 2, 2003. It was performed at 24 ± 2°C with six concentrations of test substance and a control. A 5.0 mg/L stock solution was prepared on May 30, 2003 by weighing 0.0100 g of test substance into a 2,000 mL Class A glass volumetric flask and adjusting the volume of dilution water to the line. This stock solution was mixed for approximately 10 minutes on a magnetic stirrer at room temperature, sonicated for approximately 15 minutes, and mixed on a magnetic stir plate until used. A series of solutions was prepared on May 30, 2003, by bringing 19, 38, 76, 150, 300, and 600 mL of the 5.0 mg/L stock solution to 1,000 mL with dilution water. Nominal concentrations of alpha-amylcinnamaldehyde were 0 mg/L (control), 0.095, 0.19, 0.38, 0.75, 1.5, and 3.0 mg/L. A portion of each solution was transferred into a 1.0 L glass beaker and a 1.0 L portion of dilution water was also transferred to a glass beaker to serve as a control. A stability sample, prepared with a nominal concentration of 3.0 mg/L which was not inoculated with algae, was incubated among the test vessels during the 72 hour exposure. The pH of the solutions was adjusted to 7.5 ± 0.1 with 0.1 N sodium hydroxide (VWR Lot # 2110), if required. Water quality measurements were made and each solution was inoculated with approximately 10,000 algal cells/mL.

Solutions were subdivided into 11 clear glass 40 mL vials for each treatment (the control was subdivided into 20 replicates) and the vials, which were filled to capacity to eliminate any head space, were sealed with Teflon-lined caps. Test vessels were randomly arranged on a rotary shaker adjusted to approximately 100 rpm in an incubator during the test (a random numbers table was used to select the location for each vessel). Test vessels were repositioned daily. A 24 hour light and 0 hour dark photoperiod was automatically maintained with cool-white fluorescent lights that provided a light intensity of approximately 410 to 420 footcandles (approximately 52 to 56 $\mu Ein/m^2sec$).

The number of algal cells/mL in each test vessel and the occurrence of relative size differences, unusual cell shapes, colors, flocculations, adherence of cells to test containers, or aggregation of cells was determined visually by means of direct microscopic examination with a hemocytometer. At 24, 48, and 72 hours, three treatment vessels and six control vessels were randomly selected and sacrificed (opened to the atmosphere) to allow daily determination of the number of algal cells/mL. The remaining two vessels at each concentration were used for the determination of alpha-amylcinnamaldehyde concentration at the end of the test.

Temperature of the incubator was measured and recorded daily (thermometer number 2968) and the temperature in a representative vessel of water incubated with the test vessels was continuously recorded. The pH of test solutions was measured and recorded in the single solution of each concentration prior to its distribution to test vessels at the beginning of the test, and in all test vessels used for the determination of the number of algal cells/mL at the end of the test.

A 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (2.75 mg/L alpha-amylcinnamaldehyde concentration) was combined in a 250-mL flask with 100 mL of fresh media to determine whether toxic effects were algicidal or algistatic. This culture was incubated under test conditions for 120 hours.

ANALYTICAL METHODS

Samples with nominal concentrations of 1.5 and 3.0 mg/L were diluted prior to derivitization by bringing 8 and 5 mL, respectively, to a total volume of 25 mL with dilution media. Analytical samples (approximately 10 mL of diluted and undiluted sample) were collected into 40 mL glass VOA vials that contained 2 mL of 0.01M H_2SO_4 in HPLC water and 0.5 mL of methanol. Samples were derivatized by adding 2 mL of 0.001M 2,4-dinitrophenylhydrazine in acetonitrile and 0.5 mL of concentrated phosphoric acid to each vial. The vials were capped, shaken, and allowed to sit for approximately 1 hour. A 5 mL aliquot of each derivatized sample was then brought to a total volume of 10 mL with 50/50 acetonitrile/HPLC water. Samples collected at the start of the toxicity test were collected from test solutions just prior to the addition of algae and distribution of the test solutions to sealed test vessels. Samples collected at the end of the toxicity test were centrifuged to remove algal cells from pooled replicate test vessels. An aliquot of derivatized sample was

transferred into an autosampler vial and analyzed by HPLC/UV (Hewlett Packard 1100 Series HPLC). Typical analytical conditions were:

Column:	Novapak C18, 150 x 3.9 mm, 4 μm
Column Temperature:	40°C
Column Flow Rate:	1.0 mL/minute
Run Time:	15 minutes
UV Detector Wavelength:	360 nm, Bw: 10 nm, Ref: 550 nm, Bw: 100 nm
Injection Volume:	100 μL
Retention Time:	9 ± 1 minutes
Mobile Phase:	B: acetonitrile
	A: HPLC water
Run:	Gradient with the following profile

Time	%B	%A
0.00	40	60
2.00	40	60
10.0	98	2
13.0	98	2
15.0	40	60

HPLC chromatographic quantitation was achieved using a standard curve obtained from peak areas of injections of six linearity standards: 0.0200, 0.0300, 0.0500, 0.100, 0.200, and 0.500 mg/L. Standards were prepared by serial dilution of a 1,000 mg/L standard prepared in acetonitrile. The measured concentrations of alpha-amylcinnamaldehyde in test samples were determined using the following equation:

$$\text{Concentration of Analyte} = \frac{\text{Sample Response - Curve Intercept}}{\text{Slope}} \times \text{Dilution Factor}$$

The analytical method was validated (MV1753) in triplicate at 0.050, 1.0, and 3.0 mg/L in a representative dilution water. Measured concentrations for samples with a nominal concentration of 0.050 mg/L averaged 0.0536 ± 0.00565 mg/L before centrifugation to remove any undissolved test substance and 0.0510 ± 0.00153 mg/L after centrifugation. Measured concentrations for samples with a nominal concentration of 1.0 mg/L averaged 1.01 ± 0.0153 mg/L before centrifugation and 0.932 ± 0.104 mg/L after centrifugation. Measured concentrations for samples with a nominal concentration of 3.0 mg/L averaged 2.71 ± 0.0808 mg/L before centrifugation and 2.51 ± 0.0458 mg/L after centrifugation.

The limit of quantitation (LOQ) was calculated as ten times the mean sample height converted to concentration using the amount/height of the lowest concentration calibration standard, also incorporating the dilution factor for the control samples. The LOQ during the definitive test was 0.00259 mg/L. The limit of detection (LOD) is calculated as three times the mean sample height converted to concentration using the amount/height of the lowest concentration calibration standard. The LOD during the definitive test was 0.000776 mg/L.

STATISTICAL METHODS

The average specific growth rate was calculated as the natural log of the number of cells/mL at time t_1 minus the natural log of the number of cells/mL at time t_0 divided by the exposure period. The area under the growth curve was calculated using the following formula:

$$\text{Area} = \frac{N_1-N_0}{2} \times t_1 + \frac{N_1+N_2-2N_0}{2} \times (t_2-t_1) + \frac{N_{n-1}+N_n-2N_0}{2} \times (t_n-t_{n-1})$$

where:

N_0 = nominal number of cells/mL at time t_0
N_1 = measured number of cells/mL at time t_1
N_n = measured number of cells/mL at time t_n
t_1 = time of first measurement after the beginning of the test
t_n = time of n^{th} measurement after the beginning of the test

The 24 hour EC50 values could not be calculated because there was not sufficient growth across all tested concentrations. The 48 and 72 hour EC50 values were calculated using the weighted least squares non-linear regression estimation procedure (Bruce and Versteeg, 1992). The slope of the concentration-response curve could not be calculated by this method.

The no observed effect concentration (NOEC) was determined using a one-way analysis of variance (ANOVA) and Bonferroni's test (TOXSTAT 3.3; Gulley, et al., 1990). The effective concentrations and NOECs were determined using the mean measured concentration of alpha-amylcinnamaldehyde and the number of cells/mL, average specific growth rate, and area under the growth curve.

IX. RESULTS

Insoluble material was not observed during the test. Nominal concentrations of alpha-amylcinnamaldehyde were: 0 mg/L (control), 0.095, 0.19, 0.38, 0.75, 1.5, and 3.0 mg/L. Initial measured concentrations of alpha-amylcinnamaldehyde were: ND (none detected at or above the limit of quantitation; control), 0.0934, 0.154, 0.363, 0.651, 1.39, and 2.75 mg/L. These initial measured concentrations ranged from 81 to 98% of nominal concentrations. Measured concentrations for samples collected at the end of the definitive test ranged from less than 0.00259 mg/L to 0.0176 mg/L. Initial measured concentrations were used for all calculations.

The algal population grew at an acceptable rate in the sealed vessels with no head space, resulting in an average of 312,000 cells/mL in the control after 72 hours (Table 4). No effects (relative size differences, unusual cell shapes, colors, flocculations, adherence of cells to test containers, or aggregation of cells) were noted during the test. Water quality throughout the test was within acceptable limits. The incubator temperature ranged from 23.7 to 24.0°C (Table A.1). The pH was not affected by the test substance (Table A.2).

Results of the exposure of algae to alpha-amylcinnamaldehyde for 72 hours are presented in Tables 4, 5, and 6, and cell growth during the test is illustrated in Figure 1. The 24, 48, and 72 hour EC50 values are presented in Table 7. Exposure of *Selenastrum capricornutum* to alpha-amylcinnamaldehyde for 72 hours resulted in an EC50 of 1.88 mg/L when calculated using the average specific growth rate, 1.18 mg/L when calculated using the number of cells/mL, and 1.24 mg/L when calculated using the area under the growth curve. The 72 hour NOEC is 0.154 mg/L alpha-amylcinnamaldehyde when determined using the number of cells/mL, the average specific growth rate, or the area under the growth curve.

At the conclusion of the definitive toxicity test, a 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (the 2.75 mg/L alpha-amylcinnamaldehyde concentration) was combined with 100 mL of fresh media in a 250-mL flask. The culture was incubated under test conditions for 120 hours. During this period the number of algal cells increased from an initial calculated concentration of approximately 300 cells/mL to approximately 140,000 cells/mL, indicating that the toxic effects were algistatic rather than algicidal.

Table 3. Measured concentrations of alpha-amylcinnamaldehyde in test media during the toxicity test with the freshwater alga, *Selenastrum capricornutum*.

Nominal Concentration of Alpha-Amylcinnamaldehyde (mg/L)	Measured Concentration of Alpha-Amylcinnamaldehyde (mg/L)			
	0 hour	Percent of Nominal	72 hour	Percent of Nominal
0 (control)	ND[1]	--	ND	--
0.095	0.0934	98	ND	--
0.19	0.154	81	ND	--
0.38	0.363	96	0.00483[4]	1
0.75	0.651	87	0.0176[4]	2
1.5	1.39	93	<0.00809	<1
3.0	2.75	92	0.131[4]	4
Laboratory Control Samples[2]				
0.75	0.668	89	0.591	79
	0.666	89	0.611	81
Stability Samples[3]				
3.0	2.64	88	0.290	10
Blank				
0	ND	--	ND	--

[1] ND = none detected at or above the limit of quantitation.
[2] Standards prepared in dilution water.
[3] Sample incubated with test vessels, not inoculated with algae.
[4] Measured values are below the lowest calibration standard and are therefore estimates.

Table 4. Cell growth data from the toxicity test with alpha-amylcinnamaldehyde and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Alpha-Amylcinnemaldehyde (mg/L)	Replicate[2]	Number of Cells per Milliliter[1] Hour of Exposure 0	24	48	72
Control	1	10,000	13,000	54,000	266,000
	2	10,000	11,000	58,000	418,000
	3	10,000	16,000	46,000	292,000
	4	10,000	15,000	48,000	264,000
	5	10,000	12,000	44,000	334,000
	6	10,000	16,000	52,000	300,000
	mean	10,000	14,000	50,000	312,000
0.0934	1	10,000	10,000	42,000	320,000
	2	10,000	12,000	54,000	312,000
	3	10,000	<10,000	54,000	318,000
	mean	10,000	<11,000	50,000	317,000
	% control	100	<79	100	102
0.154	1	10,000	19,000	42,000	294,000
	2	10,000	10,000	40,000	348,000
	3	10,000	<10,000	38,000	288,000
	mean	10,000	<13,000	40,000	310,000
	% control	100	<93	80	99
0.363	1	10,000	11,000	66,000	226,000
	2	10,000	<10,000	40,000	256,000
	3	10,000	10,000	42,000	214,000
	mean	10,000	<10,000	49,000	232,000
	% control	100	<71	98	74
0.651	1	10,000	<10,000	32,000	222,000
	2	10,000	11,000	54,000	260,000
	3	10,000	<10,000	30,000	280,000
	mean	10,000	<10,000	39,000	254,000
	% control	100	<71	78	81
1.39	1	10,000	12,000	34,000	124,000
	2	10,000	13,000	32,000	132,000
	3	10,000	12,000	28,000	114,000
	mean	10,000	12,000	31,000	123,000
	% control	100	86	62	39
2.75	1	10,000	10,000	12,000	17,000
	2	10,000	10,000	12,000	22,000
	3	10,000	<10,000	18,000	20,000
	mean	10,000	<10,000	14,000	20,000
	% control	100	<71	28	6

[1] Cell counts at 0 hour are calculated based on the cell density of the culture used to inoculate the test vessels. Cell counts from 24 through 72 hours were made using a hemocytometer.

[2] Replicates (20 controls and 11 per treatment) were changed at each interval.

Table 5. Average specific growth rate and percent of control from the toxicity test with alpha-amylcinnamaldehyde and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Alpha-Amylcinnamaldehyde (mg/L)		Average Specific Growth Rate		
		Hour of Exposure		
		24	48	72
Control	mean	0.0140	0.0335	0.0478
0.0934	mean	0.0040	0.0335	0.0480
	% control	28	100	100
0.154	mean	0.0109	0.0289	0.0477
	% control	78	86	100
0.363	mean	0	0.0331	0.0437
	% control	0	99	91
0.651	mean	0	0.0284	0.0449
	% control	0	85	94
1.39	mean	0.0076	0.0236	0.0349
	% control	54	70	73
2.75	mean	0	0.0070	0.0096
	% control	0	21	20

Table 6. Area under the growth curve and percent of control from the toxicity test with alpha-amylcinnamaldehyde and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Alpha-Amylcinnamaldehyde (mg/L)		Area Under the Growth Curve		
		Hour of Exposure		
		24	48	72
Control	mean	48,000	576,000	4,680,000
0.0934	mean	12,000	504,000	4,668,000
	% control	25	88	100
0.154	mean	36,000	432,000	4,392,000
	% control	75	75	94
0.363	mean	0	468,000	3,600,000
	% control	0	81	77
0.651	mean	0	348,000	3,624,000
	% control	0	60	77
1.39	mean	24,000	300,000	1,908,000
	% control	50	52	41
2.75	mean	0	48,000	216,000
	% control	0	8	5

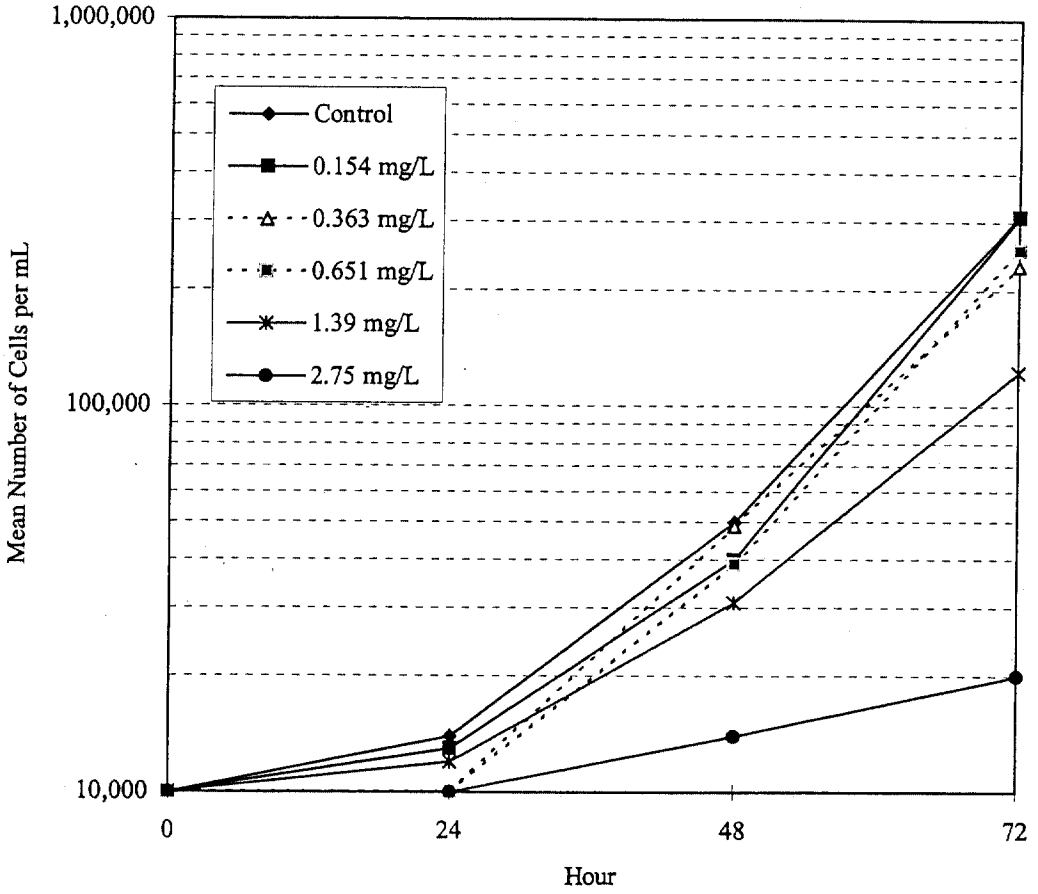

Figure 1. Growth of the freshwater alga, *Selenastrum capricornutum*, during the toxicity test with alpha-amylcinnamaldehyde.

Table 7. Median effective concentrations (EC50s) and no observed effect concentrations (NOECs) from the toxicity test with alpha-amylcinnamaldehyde and the freshwater alga, *Selenastrum capricornutum.*

Time (hours)	Value (mg/L)[1]	95 Percent Confidence Limits (mg/L)[1]
Calculated Using the Number of Cells per Milliliter		
24 EC50	---	---
48 EC50	1.82	1.38 to 2.41
72 EC50	1.18	1.03 to 1.36
72 NOEC	0.154	
Calculated Using the Average Specific Growth Rate		
24 EC50	---	---
48 EC50	1.89	1.58 to 2.25
72 EC50	1.88	1.79 to 1.97
72 NOEC	0.154	
Calculated Using the Area Under the Growth Curve		
24 EC50	---	---
48 EC50	1.58	1.20 to 2.09
72 EC50	1.24	1.09 to 1.41
72 NOEC	0.154	

[1] Based on initial measured concentrations of alpha-amylcinnamaldehyde.

X. PROTOCOL DEVIATIONS

The analytical method validation was not completed prior to the start of the definitive toxicity test (final method validation samples were analyzed with the 0 hour samples from the definitive test). This deviation had no effect on the outcome or validity of the study and no other protocol deviations occurred.

XI. SIGNATURE PAGE

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XII. REFERENCES

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OECD. 1997. OECD Principles of Good Laboratory Practice. [C(97) 186 / Final].

OECD. 1984. OECD Guidelines for Testing of Chemicals. Section 2: Effects on Biotic Systems. Method 201, Alga Growth Inhibition Test. Adopted 7 June 1984.

U.S. EPA. 1978. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test. EPA-600/9-78-018. Environmental Research Laboratory, Corvallis, Oregon.

Appendix A. WATER QUALITY DATA FROM THE TOXICITY TEST

Table A.1. Temperatures measured during the toxicity test with alpha-amylcinnamaldehyde and the freshwater alga, *Selenastrum capricornutum*.

Hour of Exposure	Temperature of Incubator (°C)
0	23.7
24	24.0
48	23.9
72	23.7

Table A.2. pH values measured during the toxicity test with alpha-amylcinnamaldehyde and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Alpha-Amylcinnamaldehyde (mg/L)	Replicate[1]	pH Initial	pH Final
Control	1	7.6	9.0
	2		9.3
	3		9.2
	4		9.2
	5		9.2
	6		9.3
0.0934	1	7.5	9.4
	2		9.4
	3		9.5
0.154	1	7.6	9.5
	2		9.5
	3		9.5
0.363	1	7.6	9.5
	2		9.6
	3		9.5
0.651	1	7.6	9.5
	2		9.6
	3		9.6
1.39	1	7.6	9.3
	2		9.2
	3		9.2
2.75	1	7.6	--[2]
	2		8.2
	3		7.9

[1] Initial pH measurements were made in stock solutions prior to their distribution into replicate test vessels. Final pH measurements were made in test vessel replicates used for algal cell counting at 72 hours (6 of the 20 control vessels and three of the 11 vessels of each treatment).

[2] Sample lost prior to measurement.

Appendix B. CERTIFICATE OF ANALYSIS

International Flavors & Fragrances Inc.

IFF

Certificate of Analysis

Ship To / Sold To:

Customer Order:
IFF Order / Item:
Delivery / Item:
Customer Number:

Info Text:

Material: **AMYLCINNAMIC ALDEHYDE**
Flashpoint: **200° F**

IFF Batch: **248084**
Order Quantity: **1 OZ** Date of Manufacture: **10/25/02**

Test	Test Data	Unit
Visual Color & Appearance	--- **VISCOLOR PASS**	
Primary Odor Evaluation	--- **ODOREVAL PASS**	
SPECIFIC GRAVITY @ D25/25	1.1161	gm/ml
SPECIFIC GRAVITY @ D20/20	1.1191	gm/ml
RELATIVE DENSITY @ D20/4	1.1171	#
REFRACTIVE INDEX @ nD25	1.5711	#
REFRACTIVE INDEX @ nD20	1.5731	#

John McGrogan
Quality Control Department

IFF Certifies that the quality of this product conforms to our product specifications.

MR# 274665

Study Title

Growth and Reproduction Toxicity Test with Nonanal and the Freshwater Alga, *Selenastrum capricornutum*

Guideline Number

OECD 201

Sponsor

The Flavor and Fragrance High Production Volume Consortia
1620 I Street, N.W.
Suite 925
Washington, DC 20006

Authors

Timothy J. Ward
Robert L. Boeri

Study Initiated

March 18, 2003

Study Completed

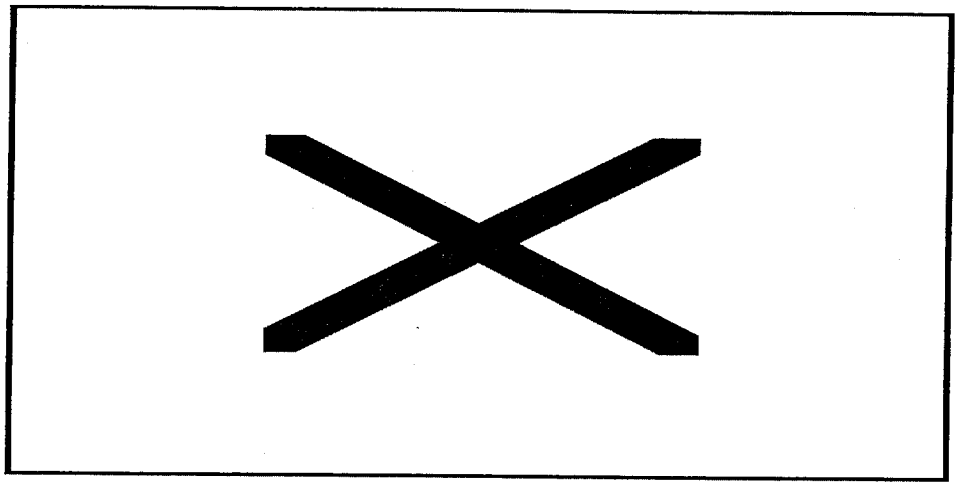
November 25, 2003

Testing Facility

T.R. Wilbury Laboratories, Inc.
40 Doaks Lane
Marblehead, Massachusetts 01945

I. GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was performed in compliance with OECD (1997) Good Laboratory Practice Standards. The test substance was used past its expiration date. Characterization of the test substance was not conducted in compliance with GLP rules.

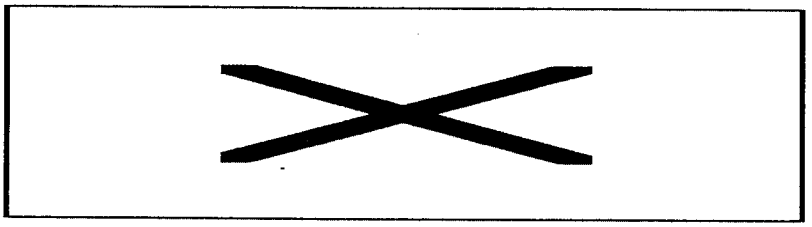


II. QUALITY ASSURANCE STATEMENT

Submitted by: T.R. Wilbury Laboratories, Inc.
40 Doaks Lane
Marblehead, Massachusetts 01945

T.R. Wilbury Laboratories, Inc., study number 2468-FF, "Growth and Reproduction Limit Toxicity Test with Nonanal and the Freshwater Alga, *Selenastrum capricornutum*," was audited by the T.R. Wilbury Quality Assurance Unit for compliance with the protocol, standard operating procedures, and applicable Good Laboratory Practices (OECD, 1997). Quality assurance audits were performed and the findings reported to the T.R. Wilbury Laboratories management and the study director on the following dates:

	Audit Date	Reported to Study Director	Reported to Management
Protocol:	29MAY02	29MAY02	29MAY02
In-life:	27JUN03	27JUN03	27JUN03
Raw data/Draft:	29JUL03	29JUL03	29JUL03
Final report:	25NOV03	25NOV03	25NOV03



III. TABLE OF CONTENTS

IV. INDEX OF TABLES AND FIGURES

V. SUMMARY

The acute toxicity of nonanal to the freshwater alga, *Selenastrum capricornutum*, is described in this report. The test was performed for 72 hours from June 27 to 30, 2003, at T.R. Wilbury Laboratories, Inc., in Marblehead, Massachusetts. It was conducted for The Flavor and Fragrance High Production Volume Consortia according to the protocol developed for T.R. Wilbury Study Number 2468-FF. Nonanal was supplied by Firmenich, Inc., 250 Plainsboro Road, Plainsboro, New Jersey, 08536.

The test was performed under static conditions in sealed containers from which all air space had been removed to minimize the potential loss of test substance from test solutions to the atmosphere. The test was run with six concentrations of test substance at 24 ± 2°C. Nominal concentrations of nonanal were 0 mg/L (control), 0.25, 0.50, 1.0, 2.0, 4.0, and 8.0 mg/L. Initial measured concentrations of nonanal were: ND (none detected at or above the limit of quantitation; control), 0.196, 0.453, 0.759, 1.47, 3.20, and 6.41 mg/L. These initial measured concentrations, which ranged from 74 to 91% of the nominal concentrations, were used to calculate median effective concentrations (EC50s). Final measured concentrations were <1 to 5% of nominal concentrations. Insoluble material was not observed at any tested concentration during the test.

The dilution water was sterile freshwater AAP medium adjusted to a pH of 7.5 ± 0.1. The test was performed in clear glass 40 mL vials that were filled to capacity to eliminate any head space and sealed with Teflon-lined caps. Algae used in the test were from a culture originally obtained from the Culture Collection of Algae of the University of Texas at Austin and acclimated to test conditions for more than 14 days at T.R. Wilbury Laboratories. Algae were distributed among twenty replicates of each control and 11 replicates of each treatment at the rate of approximately 10,000 cells/mL.

Exposure of algae to nonanal for 72 hours resulted in a median effective concentration (EC50) of 4.50 mg/L when calculated using the average specific growth rate, 2.60 mg/L when calculated using the number of cells/mL, and 1.79 mg/L when calculated using the area under the growth curve. The 72 hour no observed effect concentration (NOEC) is 0.759 mg/L nonanal when determined using the number of cells/mL, the average specific growth rate, or the area under the growth curve.

At the conclusion of the definitive toxicity test, a 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (the 3.20 and 6.41 mg/L nonanal concentrations) was combined with fresh media. These cultures were incubated under test conditions for 72 hours. During this period the number of algal cells increased from an initial concentration of 1,600 cells/mL to approximately 580,000 cells/mL at 3.20 mg/L and from 330 cells/mL to 198,000 cells/mL at 6.41 mg/L, indicating that the toxic effects were algistatic rather than algicidal.

VI. GENERAL INFORMATION

Material Tested:	Nonanal
Lot Number:	2F4107
Percent Active Ingredient:	98.0%
Study Initiation Date:	March 18, 2003 (protocol signed)
Experimental Start Date:	March 19, 2003
Experimental Termination Date:	July 8, 2003
Study Completion Date:	November 25, 2003
Archive Location -- biological raw data and a copy of the final report:	T.R. Wilbury Laboratories, Marblehead, MA
original final report	Transferred to Sponsor

VII. OBJECTIVE

The purpose of this study was to determine the 24, 48, and 72 hour median effective concentration (EC50) values and the 72 hour no observed effect concentration (NOEC) of the test substance to algae exposed under static conditions.

VIII. METHODS AND MATERIALS

A. TEST SUBSTANCE

The sample of nonanal used during the study (T.R. Wilbury sample number 1764) was delivered on March 12, 2003. It was contained in 1-L amber glass bottle that was labeled with the following information: "Aldehyde, C9, 907010, PO Nbr: S.O.97543BFS, Lot: 2F4107, Flash Point Equals 162°F, Best Before:3/2003, Production Date: 9/2002."

Nonanal (a clear liquid) was shipped from Firmenich, Inc., 250 Plainsboro Road, Plainsboro, New Jersey, 08536, at ambient temperature. The purity of the test substance was reported to be 98.0% active ingredient and the test substance was used as-received (no correction for purity). Prior to use the test substance was stored at room temperature in the dark. All unused test substance is returned to the sponsor. The stability of the test substance under test conditions (aqueous solutions in sealed containers with no head space) was determined by the analysis of samples during the definitive toxicity test.

B. DILUTION WATER

Water used for acclimation of test organisms and for all toxicity testing was sterile freshwater AAP medium (U.S. EPA, 1978; T.R. Wilbury Standard Operating Procedure number 6) at a pH of 7.5 ± 0.1. Characterization of a representative sample of deionized water used to formulate media is presented in Table 1 and a description of the media is presented in Table 2.

C. TEST ORGANISM

Algae used for the test (*Selenastrum capricornutum*, UTEX 1648) were from a culture originally procured from the Culture Collection of Algae at the University of Texas at Austin and delivered to T.R. Wilbury Laboratories on July 17, 2001. The culture was transferred to sterile enriched media identical to media used for this test and maintained at test conditions for at least 14 days before the definitive test.

During the acclimation period, the culture was actively growing in at least 2 subcultures prior to the start of the toxicity test. The subsample of algae used to inoculate media at the start of the definitive test came from an eight day old culture. Identification of the culture organisms, which are also referred to as *Raphidocelis subcapitata* and *Pseudokirchneriella subcapitata*, was verified using an appropriate taxonomic key.

Table 1. Chemical characterization of a representative sample of deionized water used to formulate dilution water for the toxicity test with nonanal and the freshwater alga, *Selenastrum capricornutum*.

Parameter[1]	Unit of Measurement	Detection Limit	Measured Value
Metals			
Aluminum	mg/L	0.01	0.02
Arsenic	mg/L	0.0004	ND[2]
Boron	mg/L	0.002	ND
Cadmium	mg/L	0.0001	ND
Calcium	mg/L	0.5	ND
Chromium	mg/L	0.0004	ND
Cobalt	mg/L	0.0001	0.0034
Copper	mg/L	0.0006	ND
Iron	mg/L	0.005	ND
Lead	mg/L	0.0001	0.001
Magnesium	mg/L	0.01	ND
Mercury	mg/L	0.0001	ND
Nickel	mg/L	0.0001	ND
Potassium	mg/L	0.1	ND
Silver	mg/L	0.0002	ND
Sodium	mg/L	0.5	ND
Zinc	mg/L	0.002	0.010
Chloride	mg/L	0.1	ND
Fluoride	mg/L	0.05	ND
Total Phosphorus	mg/L	0.02	ND
Total Organic Carbon	mg/L	0.5	ND
Organochlorine Pesticides	µg/L	4.0	ND
Toxaphene	µg/L	10.0	ND
Methoxychlor	µg/L	10.0	ND
Mirex	µg/L	10.0	ND
Organophosphorus Pesticides	µg/L	0.33	ND
PCBs	µg/L	0.05	ND

[1] Parameters were measured in deionized water (used to formulated dilution water) that was collected on December 24, 2002 (chloride and fluoride samples were collected on December 23, 2002) and analyzed by Enviro-Test Laboratories as part of routine water quality testing conducted twice per year.

[2] ND = not detected at or above the method detection limit.

Table 2. Description of the freshwater AAP medium used for the toxicity test with nonanal and the freshwater alga, *Selenastrum capricornutum*.

	Nutrient[1]	Final Concentration in Algal Medium (mg/L)
Macronutrients	$NaNO_3$	25.5
	$MgCl_2 \cdot 6H_2O$	12.2
	$CaCl_2 \cdot 2H_2O$	4.4
	$MgSO_4 \cdot 7H_2O$	14.7
	K_2HPO_4	1.044
	$NaHCO_3$	15.0
Micronutrients	H_3BO_3	0.1855
	$CoCl_2 \cdot 6H_2O$	0.0014
	$MnCl_2 \cdot 4H_2O$	0.4154
	$Na_2MoO_4 \cdot 2H_2O$	0.0073
	$ZnCl_2$	0.0033
	$Na_2EDTA \cdot 2H_2O$	0.30
	$FeCl_2 \cdot 6H_2O$	0.1598
	$CuCl_2 \cdot 2H_2O$	0.000012

[1] Each of the nutrients was prepared as a sterile stock solution in deionized water at 1,000 times the concentrations in the table. Algal medium was prepared by the addition of 1 mL of each stock solution to 1,000 mL of sterile deionized water.

D. TOXICITY TEST METHODS

The study was performed according to T.R. Wilbury Study Protocol 2468-FF (Growth and Reproduction Limit Toxicity Test with Nonanal and the Freshwater Alga, *Selenastrum capricornutum*), which was based on procedures of the OECD (1984). It was conducted for The Flavor and Fragrance High Production Volume Consortia and the Sponsor's Representative was Dr. Timothy B. Adams.

A range-finding test was conducted from March 19 to 22, 2003 with a control and five concentrations of nonanal. At the conclusion of the test, the percent of control growth was as follows; 0.01, 0.10, and 1.0 mg/L = >100%, 10 mg/L = 9%, and 100 mg/L = <3%. A definitive test was initiated on June 20, 2003 with a control and six concentrations of nonanal (0.50, 1.0, 2.0, 3.8, 7.5, and 15 mg/L). The test was terminated after 48 hours and repeated because analytical recoveries in samples collected at the start of the test were unacceptably low.

The final definitive toxicity test was conducted from June 27 to 30, 2003. It was performed at 24 ± 2°C with six concentrations of test substance and a control. An 8.0 mg/L stock solution was prepared on June 27, 2003 by weighing 0.0161 g of test substance into a 2,000 mL Class A glass volumetric flask and adjusting the volume of dilution water to the line. This stock solution was mixed and the pH was adjusted with 0.1 N NaOH (VWR Lot # 2110). A series of solutions was prepared on June 27, 2003, by bringing 31, 63, 130, 250, 500, and 1,000 mL of the 8.0 mg/L stock solution to 1,000 mL with dilution water. Nominal concentrations of nonanal were 0 mg/L (control), 0.25, 0.50, 1.0, 2.0, 4.0, and 8.0 mg/L. A portion of each solution was transferred into a 1.0 L glass beaker and a 1.0 L portion of dilution water was also transferred to a glass beaker to serve as a control. A stability sample, prepared with a nominal concentration of 8.0 mg/L but not inoculated with algae, was incubated among the test vessels during the 72 hour exposure. Water quality measurements were made and each solution was inoculated with approximately 10,000 algal cells/mL.

Solutions were subdivided into 11 clear glass 40 mL vials for each treatment (the control was subdivided into 20 replicates) and the vials, which were filled to capacity to eliminate any head space, were sealed with Teflon-lined caps. Test vessels were randomly arranged on a rotary shaker adjusted to approximately 100 rpm in an incubator during the test (a random numbers table was used to select the location for each vessel) and vesels were repositioned daily. A 24 hour light and 0 hour dark photoperiod was automatically maintained with cool-white fluorescent lights that provided a light intensity of approximately 400 footcandles (approximately 52 to 54 $\mu Ein/m^2sec$).

The number of algal cells/mL in each test vessel and the occurrence of relative size differences, unusual cell shapes, colors, flocculations, adherence of cells to test containers, or aggregation of cells was determined visually by means of direct microscopic examination with a hemocytometer. At 24, 48, and 72 hours, three treatment vessels and six control

vessels were randomly selected and sacrificed (opened to the atmosphere) to allow daily determination of the number of algal cells/mL. The remaining two vessels at each concentration were used for the determination of nonanal concentration at the end of the test.

Temperature of the incubator was measured and recorded daily (thermometer number 2968) and the temperature in a representative vessel of water incubated with the test vessels was continuously recorded. The pH of test solutions was measured and recorded in the single solution of each concentration prior to its distribution to test vessels at the beginning of the test, and in all test vessels used for the determination of the number of algal cells/mL at the end of the test.

A 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (3.20 and 6.41 mg/L nonanal concentrations) was combined in a 250-mL flask with 100 mL of fresh media to determine whether toxic effects were algicidal or algistatic. These cultures were incubated under test conditions for 72 hours.

ANALYTICAL METHODS

Analytical samples (approximately 10 mL) were collected into 40 mL glass VOA vials that contained 2 mL of 0.01M H_2SO_4 and 0.5 mL methanol in HPLC water. Samples were derivatized by adding 2 mL of 0.001M 2,4-dinitrophenylhydrazine in acetonitrile and 0.5 mL of concentrated phosphoric acid to each vial. The vials were capped, shaken, and allowed to derivatize for approximately 1 hour. A 5 mL aliquot of each derivatized sample was then brought to a total volume of 10 mL with 50/50 acetonitrile/HPLC water. Samples collected at the start of the toxicity test were collected from test solutions just prior to the addition of algae and distribution of the test solutions to sealed test vessels. Samples collected at the end of the toxicity test were centrifuged to remove algal cells from pooled replicate test vessels. Samples outside the calibration range were diluted into the range with 50/50 acetonitrile/HPLC water. An aliquot of derivatized sample was transferred into an autosampler vial and analyzed by HPLC/UV (Hewlett Packard 1100 Series HPLC). Typical analytical conditions were:

Column:	Novapak C18, 150 x 3.9 mm, 4 μm
Column Flow Rate:	1.0 mL/minute
Run Time:	20 minutes
UV Detector Wavelength:	360 nm, Bw: 10 nm, Ref: 550 nm, Bw: 100 nm
Injection Volume:	100 μL
Retention Time:	11.3 ± 1.0 minutes
Mobile Phase:	A: HPLC water
	B: acetonitrile

Run: Gradient at 40°C with the following profile

Time	%B	%A
0.00	40	60
2.00	40	60
10.0	98	2
15.0	98	2
16.0	40	60

HPLC chromatographic quantitation was achieved using a standard curve obtained from peak areas of injections of up to seven linearity standards: 0.050, 0.075, 0.100, 0.150, 0.200, 0.250, and 0.300 mg/L. Standards were prepared by serial dilution of a 1,000 mg/L standard prepared in acetonitrile and then derivatized in a manner identical to the samples from the toxicity test. The measured concentrations of nonanal in test samples were determined using the following equation:

$$\text{Concentration of Analyte} = \frac{\text{Sample Response - Curve Intercept}}{\text{Slope}} \times \text{Dilution Factor}$$

The analytical method was validated (T.R. Wilbury study number MV-1764) in triplicate at 0.20, 2.0, and 20 mg/L in a representative dilution water. Measured concentrations for samples with a nominal concentration of 0.20 mg/L averaged 0.160 ± 0.011 mg/L before centrifugation to remove any undissolved test substance and 0.171 ± 0.002 mg/L after centrifugation. Measured concentrations for samples with a nominal concentration of 2.0 mg/L averaged 1.61 ± 0.168 mg/L before centrifugation and 1.62 ± 0.025 mg/L after centrifugation. Measured concentrations for samples with a nominal concentration of 20 mg/L averaged 17.0 ± 0.231 mg/L before centrifugation and 15.3 ± 0.551 mg/L after centrifugation.

The limit of quantitation (LOQ) was calculated as ten times the mean sample height converted to concentration using the amount/height of the lowest concentration calibration standard, also incorporating the dilution factor for the control samples. The LOQ during the definitive test was 0.00176 mg/L. The limit of detection (LOD) is calculated as three times the mean sample height converted to concentration using the amount/height of the lowest concentration calibration standard. The LOD during the definitive test was 0.000528 mg/L.

E. STATISTICAL METHODS

The average specific growth rate was calculated as the natural log of the number of cells/mL at time t_1 minus the natural log of the number of cells/mL at time t_0 divided by the exposure period. The area under the growth curve was calculated using the following formula:

$$\text{Area} = \frac{N_1-N_0}{2} \times t_1 + \frac{N_1+N_2-2N_0}{2} \times (t_2-t_1) + \frac{N_{n-1}+N_n-2N_0}{2} \times (t_n-t_{n-1})$$

where:

N_0 = nominal number of cells/mL at time t_0
N_1 = measured number of cells/mL at time t_1
N_n = measured number of cells/mL at time t_n
t_1 = time of first measurement after the beginning of the test
t_n = time of n^{th} measurement after the beginning of the test

The 24, 48, and 72 hour EC50 values were calculated using the weighted least squares non-linear regression estimation procedure (Bruce and Versteeg, 1992), when possible (the 24 hour EC50 could not be calculated using the number of cells per mL or the area under the growth curve). The slope of the concentration-response curve could not be calculated by this method.

The no observed effect concentration (NOEC) was determined using a one-way analysis of variance (ANOVA) and Bonferroni's test (TOXSTAT 3.3; Gulley, et al., 1990). The effective concentrations and NOECs were determined using the initial measured concentration of nonanal and the number of cells/mL, average specific growth rate, and area under the growth curve.

IX. RESULTS

Insoluble material was not observed during the test. Nominal concentrations of nonanal were: 0 mg/L (control), 0.25, 0.50, 1.0, 2.0, 4.0, and 8.0 mg/L. Initial measured concentrations of nonanal were: ND (none detected at or above the limit of quantitation; control), 0.196, 0.453, 0.759, 1.47, 3.20, and 6.41 mg/L. These initial measured concentrations, which ranged from 74 to 91% of the nominal concentrations, were used to calculate median effective concentrations (EC50s). Final measured concentrations were <1 to 5% of nominal concentrations. Initial measured concentrations were used for all calculations.

The algal population grew at an acceptable rate in the sealed vessels with no head space, resulting in an average of 220,000 cells/mL in the control after 72 hours (Table 4). No effects (relative size differences, unusual cell shapes, colors, flocculations, adherence of cells to test containers, or aggregation of cells) were noted during the test. Water quality throughout the test was within acceptable limits. The incubator temperature ranged from 23.5 to 23.9°C (Table A.1). The pH was not affected by the test substance (Table A.2).

Results of the exposure of algae to nonanal for 72 hours are presented in Tables 4, 5, and 6, and cell growth during the test is illustrated in Figure 1. The 24, 48, and 72 hour EC50 values are presented in Table 7. Exposure of *Selenastrum capricornutum* to nonanal for 72 hours resulted in an EC50 of 4.50 mg/L when calculated using the average specific growth rate, 2.60 mg/L when calculated using the number of cells/mL, and 1.79 mg/L when calculated using the area under the growth curve. The 72 hour NOEC is 0.759 mg/L nonanal when determined using the number of cells/mL, the average specific growth rate, or the area under the growth curve.

At the conclusion of the definitive toxicity test, a 0.5 mL aliquot of test media from each test vessel where growth was maximally inhibited (the 3.20 and 6.41 mg/L nonanal concentrations) was combined with 100 mL of fresh media in a 250-mL flask. The cultures were incubated under test conditions for 72 hours. During this period the number of algal cells increased from an initial concentration of 1,600 cells/mL to approximately 580,000 cells/mL at 3.20 mg/L and from 330 cells/mL to 198,000 cells/mL at 6.41 mg/L, indicating that the toxic effects were algistatic rather than algicidal.

Table 3. Measured concentrations of nonanal in test media during the toxicity test with the freshwater alga, *Selenastrum capricornutum*.

Nominal Concentration of Nonanal (mg/L)	Measured Concentration of Nonanal (mg/L)			
	0 hour	Percent of Nominal	72 hour	Percent of Nominal
0 (control)	ND	--	ND	--
0.25	0.196	78	ND	--
0.50	0.453	91	<0.00440	<1
1.0	0.759	76	0.0187[4]	2
2.0	1.47	74	0.0530[4]	3
4.0	3.20	80	0.212[4]	5
8.0		80	0.373[4]	5
Laboratory Control Samples[2]				
2.0	1.74	87	1.41	71
	1.81	91	1.48	74
Stability Samples[3]				
8.0	6.54	82	5.14	64
Blank				
0	ND	--	<0.0751	--

[1] ND = none detected at or above the limit of quantitation.
[2] Standards prepared in dilution water.
[3] Sample incubated with test vessels, not inoculated with algae.
[4] Estimated value because the instrument response was less than the lowest standard.

Table 4. Cell growth data from the toxicity test with nonanal and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Nonanal (mg/L)	Replicate[2]	Number of Cells per Milliliter[1]			
		Hour of Exposure			
		0	24	48	72
Control	1	10,000	27,000	88,000	214,000
	2	10,000	20,000	94,000	204,000
	3	10,000	24,000	100,000	196,000
	4	10,000	21,000	110,000	252,000
	5	10,000	23,000	74,000	238,000
	6	10,000	23,000	84,000	216,000
	mean	10,000	23,000	92,000	220,000
0.196	1	10,000	20,000	66,000	238,000
	2	10,000	21,000	84,000	266,000
	3	10,000	18,000	80,000	216,000
	mean	10,000	20,000	77,000	240,000
	% control	100	87	84	109
0.453	1	10,000	27,000	108,000	236,000
	2	10,000	23,000	74,000	218,000
	3	10,000	28,000	86,000	220,000
	mean	10,000	26,000	89,000	225,000
	% control	100	113	97	102
0.759	1	10,000	18,000	84,000	248,000
	2	10,000	13,000	116,000	234,000
	3	10,000	16,000	60,000	266,000
	mean	10,000	16,000	87,000	249,000
	% control	100	70	95	113
1.47	1	10,000	12,000	14,000	152,000
	2	10,000	16,000	20,000	154,000
	3	10,000	11,000	28,000	148,000
	mean	10,000	13,000	21,000	151,000
	% control	100	57	23	69
3.20	1	10,000	10,000	22,000	122,000
	2	10,000	16,000	28,000	86,000
	3	10,000	19,000	24,000	110,000
	mean	10,000	15,000	25,000	106,000
		100	65	27	48
6.41	1	10,000	15,000	<10,000	24,000
	2	10,000	14,000	<10,000	29,000
	3	10,000	10,000	<10,000	14,000
	mean	10,000	13,000	<10,000	22,000
		100	57	<11	10

[1] Cell counts at 0 hour are calculated based on the cell density of the culture used to inoculate the test vessels. Cell counts from 24 through 72 hours were made using a hemocytometer.

[2] Replicates (20 controls and 11 per treatment) were changed at each interval.

Table 5. Average specific growth rate and percent of control from the toxicity test with nonanal and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Nonanal (mg/L)		Average Specific Growth Rate		
		Hour of Exposure		
		24	48	72
Control	mean	0.035	0.046	0.043
0.196	mean	0.029	0.043	0.044
	% control	83	93	102
0.453	mean	0.040	0.046	0.043
	% control	114	100	100
0.759	mean	0.020	0.045	0.045
	% control	57	98	105
1.47	mean	0.011	0.015	0.038
	% control	31	33	88
3.20	mean	0.017	0.019	0.033
	% control	49	41	77
6.41	mean	0.011	0.000	0.011
	% control	31	0	26

Table 6. Area under the growth curve and percent of control from the toxicity test with nonanal and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Nonanal (mg/L)		Area Under the Growth Curve		
		Hour of Exposure		
		24	48	72
Control	mean	156,000	1,296,000	4,800,000
0.196	mean	120,000	1,044,000	4,608,000
	% control	77	81	96
0.453	mean	192,000	1,332,000	4,860,000
	% control	123	103	101
0.759	mean	72,000	1,068,000	4,860,000
	% control	46	82	101
1.47	mean	36,000	204,000	2,028,000
	% control	23	16	42
3.20	mean	60,000	300,000	1,632,000
	% control	38	23	34
6.41	mean	36,000	72,000	216,000
	% control	23	6	5

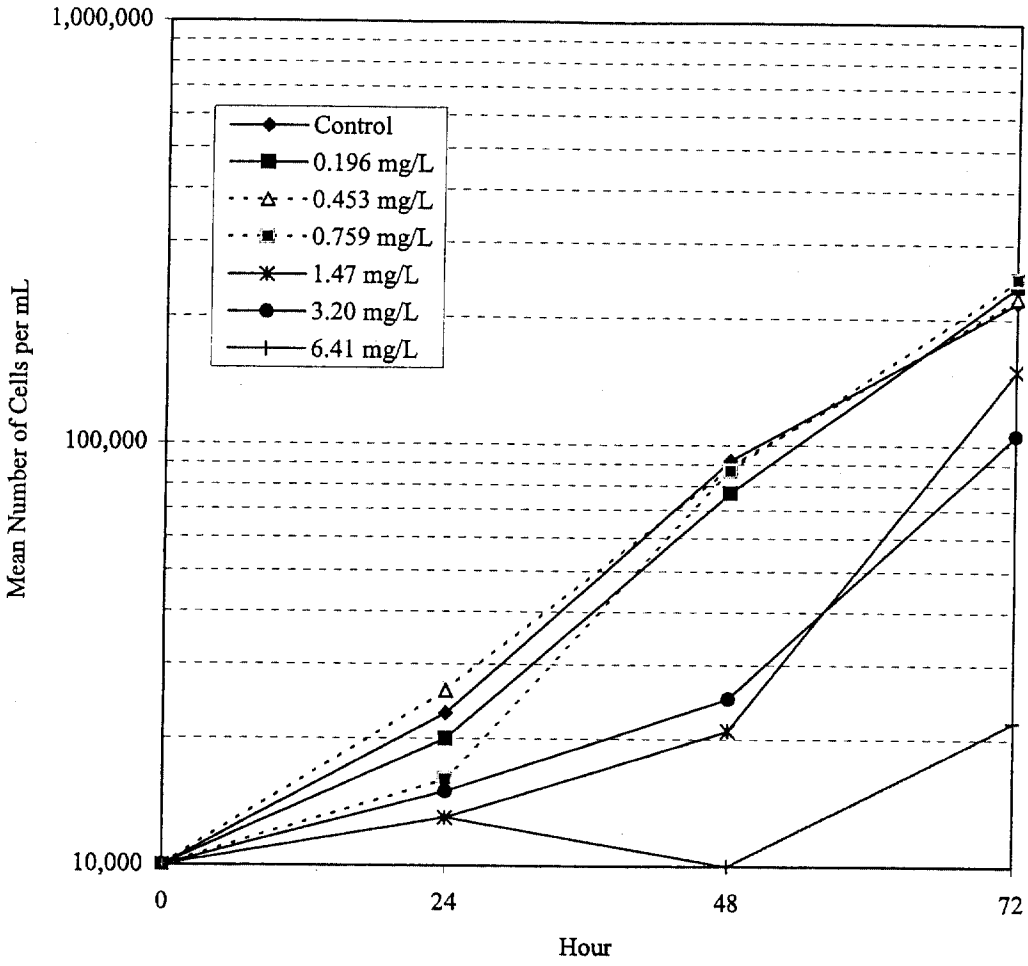


Figure 1. Growth of the freshwater alga, *Selenastrum capricornutum*, during the toxicity test with nonanal.

Table 7. Median effective concentrations (EC50s) and no observed effect concentrations (NOECs) from the toxicity test with nonanal and the freshwater alga, *Selenastrum capricornutum*.

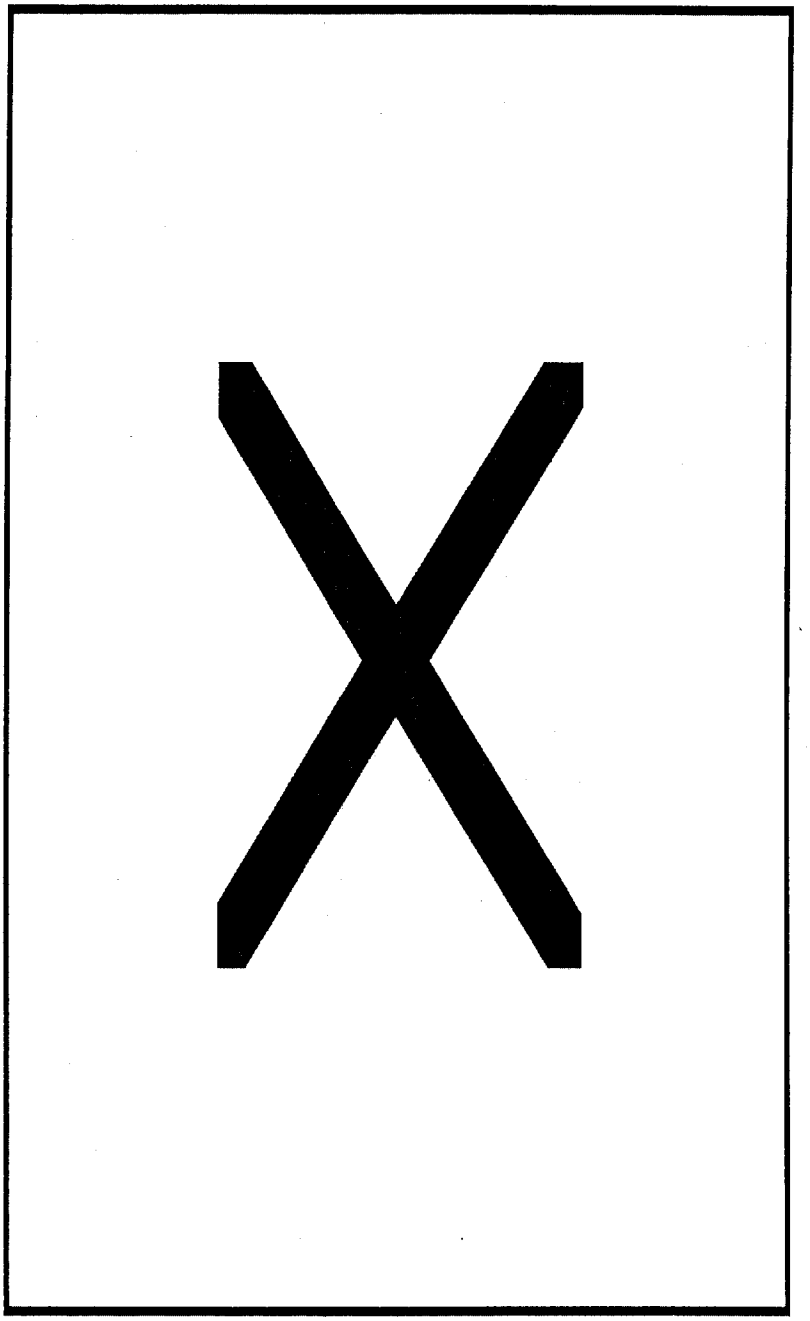
Time (hours)	Value (mg/L)[1]	95 Percent Confidence Limits (mg/L)[1]
Calculated Using the Number of Cells per Milliliter		
24 EC50	>6.41	
48 EC50	1.40	0.779 to 2.52
72 EC50	2.60	2.17 to 3.11
72 NOEC	0.759	
Calculated Using the Average Specific Growth Rate		
24 EC50	1.60	0.333 to 7.70
48 EC50	1.73	1.11 to 2.70
72 EC50	4.50	4.03 to 5.02
72 NOEC	0.759	
Calculated Using the Area Under the Growth Curve		
24 EC50	1.24	0.256 to 5.98
48 EC50	1.17	0.651 to 2.12
72 EC50	1.79	1.36 to 2.37
72 NOEC	0.759	

[1] Based on initial measured concentrations of nonanal.

X. PROTOCOL DEVIATIONS

No protocol deviations occurred.

XI. SIGNATURE PAGE



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Appendix A. WATER QUALITY DATA FROM THE TOXICITY TEST

Table A.1. Temperatures measured during the toxicity test with nonanal and the freshwater alga, *Selenastrum capricornutum*.

Hour of Exposure	Temperature of Incubator (°C)
0	23.5
24	23.6
48	23.7
72	23.9

Table A.2. pH values measured during the toxicity test with nonanal and the freshwater alga, *Selenastrum capricornutum*.

Initial Measured Concentration of Nonanal (mg/L)	Replicate[1]	pH	
		Initial	Final
Control	1	7.4	9.4
	2		9.5
	3		9.6
	4		9.8
	5		9.8
	6		9.6
0.196	1	7.5	9.8
	2		9.8
	3		9.9
0.453	1	7.5	9.8
	2		9.9
	3		9.9
0.759	1	7.5	9.9
	2		9.8
	3		9.7
1.47	1	7.6	9.3
	2		9.3
	3		9.3
3.20	1	7.5	8.5
	2		8.7
	3		8.7
6.41	1	7.5	7.1
	2		7.1
	3		7.1

[1] Initial pH measurements were made in stock solutions prior to their distribution into replicate test vessels. Final pH measurements were made in test vessel replicates used for

algal cell counting at 72 hours (6 of the 20 control vessels and three of the 11 vessels of each treatment).

Appendix B. CERTIFICATE OF ANALYSIS